Glucagon-like peptide-1 receptor agonist, semaglutide attenuates chronic liver disease-induced skeletal muscle atrophy in diabetic mice

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Abstract

A glucagon-like peptide-1 receptor agonist (GLP-1RA) has recently been established as a pharmacological option for the treatment of type 2 diabetes. Recent studies have demonstrated the molecular role of GLP-1R in skeletal muscle homeostasis; however, the therapeutic efficacy of semaglutide, a GLP-1RA, on skeletal muscle atrophy in chronic liver disease (CLD) under diabetic conditions remains unclear. In the present study, semaglutide effectively inhibited psoas muscle atrophy and suppressed declines in grip strength in a diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet-fed diabetic KK-A^y mouse model. Moreover, semaglutide inhibited ubiquitin-proteosome-mediated skeletal muscle proteolysis and promoted myogenesis in palmitic acid (PA)-stimulated C2C12 murine myocytes. Mechanistically, this effect of semaglutide on skeletal muscle atrophy was mediated by multiple functional pathways. First, semaglutide protected against hepatic injury in mice accompanied by increased production of insulin-like growth factor 1 and reduced accumulation of reactive oxygen species (ROS). These effects were associated with decreased proinflammatory cytokines and ROS accumulation, leading to the suppression of ubiquitin-proteosome muscle degradation. Moreover, semaglutide inhibited the amino acid starvation-related stress signaling that was activated under chronic liver injury, resulting in the recovery of the mammalian target of rapamycin activity in the skeletal muscle of DDC-diet fed KK-A^y mice. Second, semaglutide improved skeletal muscle atrophy by directly stimulating GLP-1R in myocytes. Semaglutide induced cAMP-mediated activation of PKA and AKT, enhanced mitochondrial biogenesis, and reduced ROS accumulation, thereby resulting in inhibition of NF-κB/myostatin-mediated ubiquitin-proteosome degradation and the augmentation of heat-shock factor-1-mediated myogenesis. Collectively, semaglutide may have potential as a new therapeutic

strategy for CLD-related skeletal muscle wasting.

Keywords: type 2 diabetes, semaglutide, sarcopenia, liver cirrhosis

Abbreviations

CLD: chronic liver disease

CT: computed tomography

DAPI: 4',6-diamidino-2-phenylindole

DDC: diethoxycarbonyl-1,4-dihydrocollidine

DMEM: Dulbecco's Modified Eagle's Medium

Ex-9: exendin-9

GLP-1RA: glucagon-like peptide-1 receptor agonist

H&E: hematoxylin and eosin

4-HNE: 4-hydroxynonenal

MDA: malondialdehyde

MSTN: myostatin

mtTFA: mitochondrial transcription factor A

MuRF-1: muscle RING-finger protein-1

MyoG: myogenin

ND: Normal diet

NF-κB: nuclear factor-κB

NRF2: nuclear factor erythroid 2-related factor 2

PA: palmitic acid

PBS: phosphate-buffered saline

PGC-1α: peroxisome proliferator-activated receptor γ coactivator-1α

PKA C-α: protein kinase A catalytic-α

PMI: psoas muscle mass index

ROS: reactive oxygen species

UPS: ubiquitin-proteasome system

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1. Introduction

Sarcopenia, defined as the progressive loss of muscle mass and characterized by reduced muscle strength and physical function, is attributed to a state of advanced malnutrition [1]. Sarcopenia often develops as an age-related condition in elderly individuals and is associated with serious health outcomes, including reduced mobility, reduced quality of life, and increased mortality [2]. It is also associated with many acute and chronic illnesses, such as type 2 diabetes and chronic liver disease (CLD) [3,4], and is recognized as a representative complication in patients with liver cirrhosis, with a reported prevalence of 30%–70% [5]. Numerous factors play a role in the pathogenesis of malnutrition and sarcopenia in patients with CLD, such as inadequate dietary intake, poor appetite, and dysgeusia, in addition to impaired protein metabolism [4,5]. Moreover, hyperammonemia has been posited to be a key driver of cirrhosis-associated sarcopenia through several molecular signaling pathways [6]. Accordingly, there is increasing clinical demand for novel therapeutic agents targeting sarcopenia in patients with CLD. Although physical exercise and nutritional supplementation are recommended for sarcopenia in patients with CLD, the efficacy of these therapies are limited [4,7].

Glucagon-like peptide-1 (GLP-1), the endogenous ligand of the G protein-coupled GLP-1 receptor (GLP-1R), is an incretin hormone that regulates glucose homeostasis [8]. GLP-1 maintains pancreatic β-cell survival and stimulates insulin secretion in a glucose-dependent manner, while suppressing gastric emptying and inducing satiety, thereby preventing excessive food intake [8]. Accordingly, GLP-1 has been established as a pharmacological target for the treatment of type 2 diabetes, diabetes-related non-alcoholic fatty liver disease, and cardiovascular disease [9–11]. Notably, the GLP-1/GLP-1R axis appears to have a broad range of physiological actions. Several reports have

demonstrated the molecular role of the GLP-1/GLP-1R axis in skeletal muscle homeostasis and the therapeutic potential of GLP-1R agonists (GLP-1RA) for skeletal muscle atrophy [12,13]. Dulaglutide, a long-acting GLP-1RA, was reported to have inhibitory effects on aging-induced muscle wasting in mice [14]. A recent study on rodents also reported that exendin-4 suppresses skeletal muscle wasting in both dexamethasone-induced and chronic kidney disease-derived muscle atrophy [15]. Moreover, GLP-1RA has been shown to increase glycogenesis in the skeletal muscle of rats with type 2 diabetes and increase oxygen consumption in a murine myoblast cell line [16]. A recent clinical study demonstrated the effect of dipeptidyl peptidase 4 inhibitors on attenuating aging-related muscle mass loss in patients with type 2 diabetes [17].

Semaglutide is a recently developed GLP-1RA with a half-life of approximately one week [18]. In the USA, semaglutide has been approved for long-term weight management as an adjunct to caloric restriction and exercise in adults with obesity or coexisting obesity-related conditions [19]. Although the efficacy of semaglutide in treating obesity and type2 diabetes has been confirmed by numerous studies, the effect of semaglutide on skeletal muscle atrophy caused by CLD under diabetic conditions is yet to be elucidated.

Consequently, the aim of the present study was to investigate the effect of semaglutide on skeletal muscle wasting and dysfunction in CLD-related skeletal muscle atrophy under diabetic conditions using a diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet-fed diabetic KK-A^{y} mouse model [20].

2. Materials and methods

2.1. Animals and treatment

A total 40 male diabetic KK-Ay mice aged 10 weeks old (CLEA Japan, Tokyo,

Japan) were randomly divided into four groups (n = 10) and treated for six weeks as follows: i) normal diet (ND) and subcutaneous injection of phosphate-buffered saline (PBS) as a vehicle (ND-Veh group); ii) ND and subcutaneous injection of semaglutide (3 nmol/kg) (AdipoGen Life Sciences Co., San Diego, CA, USA) every 3 days (ND-Sem group); iii) 0.01% DDC diet (Research Diets Inc., New Brunswick, NJ, USA) and subcutaneous injection of PBS (DDC-Veh group); and iv) DDC diet and subcutaneous injection of semaglutide every three days (DDC-Sem group) [21]. After the in vivo experiment, mice were deeply anesthetized with sodium pentobarbital intraperitoneally (200 mg/kg) on a feeding day after 6 h of fasting, opening of the abdominal cavity, blood collection via puncture of the aorta, and harvesting of the liver and gastrocnemius muscle. Serum levels of glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, and bilirubin were measured using Glucose Assay kits (Abcam, Cambridge, UK), Mouse Aspartate Aminotransferase ELISA kits (Abcam), Mouse Alanine Aminotransferase ELISA kits (Abcam), Mouse Albumin ELISA kits (Abcam), and QuantiChrom™ Bilirubin Assay kits (BioAssay Systems, Hayward, CA, USA), respectively, according to the manufacturers' protocols.

All experimental studies were approved, and the methods were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80–23) revised in 2011, and all experimental procedures were approved by the Animal Ethics Committee of Nara Medical University (approval no.13029).

2.2. Mouse myoblast culture

C2C12 mouse myoblasts (RCB0987, RIKEN BRC Cell Bank, Ibaragi, Japan) were seeded in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque,

Kyoto, Japan) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% penicillin/streptomycin at 37°C with 5% CO_2. To differentiate them into myotubes, C2C12 myoblasts were further cultured in DMEM supplemented with 2% horse serum for 8 days. After differentiation into myotubes (60%–70%) based on morphological alignment, elongation, and fusion, these cells were used for in vitro assays [22]. To induce oxidative stress, the differentiated C2C12 cells were stimulated with palmitic acid (PA) (Nacalai Tesque) at varying concentrations (0–1 mM) for 24 h. C2C12 cells were pre-treated with semaglutide (0.3–0.9 μM) for 24 h to determine the direct effect of semaglutide on myocytes [23,24]. Moreover, C2C12 cells were pre-treated with 0.2 μM of exendin-9 (Ex-9) and GLP-1R antagonist (Abcam, Cambridge, UK) for 2 h before treatment with semaglutide to evaluate the effect of semaglutide on the GLP-1R [25].

2.3. Psoas muscle mass index (PMI) and grip strength

All mice underwent abdominal computed tomography (CT) using CosmoScan FX (Rigaku Corporation, Tokyo, Japan) before and at three and six weeks after the start of treatment [26]. Mouse PMI (cross sectional area/height^2) was calculated on a single CT slice at the level of the L3 pedicle using Slice-O-Matic (Tomovision, Montreal, Canada) [27]. Hindlimb and forelimb grip strength was simultaneously measured using a grip strength meter (MK-380Si) (Muromachi Kikai, Co. Ltd., Kyoto, Japan) as described previously [26]. Grip strength was measured before and at three and six weeks after the start of treatment, and each measurement was calculated as the average of three consecutive measurements.

2.4 Serum and hepatic levels of insulin-like growth factor 1 (IGF-1)

The serum IGF-1 concentrations in mice were measured using a Mouse/Rat IGF-I/IGF-1 Quantikine ELISA kit (R&D systems, Minneapolis, MN, USA). All samples were processed and assayed according to the manufacturer's protocol.

2.5. Hepatic and muscular levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE)

Amounts of MDA and 4-HNE in 100 mg of mouse liver tissue homogenate were determined using Lipid Peroxidation (MDA) Assay kits (Sigma-Aldrich, St. Louis, MO, USA) and Lipid Peroxidation (4-HNE) Assay kits (Abcam), respectively. The amount of 4-HNE in 10 mg of mouse gastrocnemius muscle tissue homogenate was measured using same method as used for liver tissue. All samples were processed and assayed according to the manufacturer’s protocol.

2.6. In vivo p70S6K phosphorylation in skeletal muscle

A semiquantitative measurement of p-p70S6K (Thr389) and total p70S6K in 2 mg of mouse gastrocnemius muscle tissue homogenate was performed using the p70S6K (pT389 + Total) ELISA Kit (Abcam) according to the manufacturer's instructions. The phosphorylation rate was calculated as the ratio of p-p70S6K (Thr389) to the total p70S6K.

2.7. Histological and immunofluorescent analyses

Liver and gastrocnemius muscle tissue were fixed in 10% formalin for 24 h at room temperature. The paraffin-embedded specimens were sectioned at 5 μm and subjected to hematoxylin and eosin (H&E) and Sirius Red (performed at Narabyouri Research Co., Nara, Japan). Hepatic inflammation was histologically determined as described previously [28]. The minimum Feret diameter was measured as the muscle fiber size [29]. Diameters were

categorized in 5 μm ranges. Primary antibodies, including rabbit-polyclonal anti-mouse CD45 antibody (5 μg/ml; ab10558, Abcam) and rabbit-monoclonal anti-mouse GLP-1R antibody (1:500; ab218532, Abcam), were used for the immunofluorescence analyses of liver and gastrocnemius specimens, respectively, according to the manufacturer's instructions. The primary antibody was detected using Goat anti-Rabbit IgG (H+L) Cross-Adsorbed ReadyProbes™ Secondary Antibody, Alexa Fluor™ 488 (1:200; R37116, Thermo Fisher Scientific, Inc.). 4',6-diamidino-2-phenylindole (DAPI) Fluoromount-G(R) (SouthernBiotech, Birmingham, AL, USA) was used for nuclear staining. Histologically and fluorescently stained tissues were viewed under a BX53 microscope (Olympus, Tokyo, Japan) for histology and a BZ-X700 microscope (Keyence, Osaka, Japan), respectively. All slides were photographed and five images were randomly selected and analyzed using ImageJ 64-bit Java 1.8.0. (National Institute of Health, Bethesda, MD, USA).

2.8. RNA isolation and real-time quantitative PCR

Total RNA isolation was performed using RNeasy Mini kits (Qiagen, Hilden, Germany) for mouse liver tissues and cultured C2C12 myotubes and using RNeasy Fibrous Tissue Mini kits (Qiagen) for mouse muscle tissues. Reverse transcription was performed using High-Capacity RNA-to-cDNA kits (Thermo Fisher Scientific, Inc.). Real-time qPCR was performed using a SYBR™-Green PCR Master Mix (Thermo Fisher Scientific, Inc.) and an Applied Biosystems StepOnePlus™ Real-Time PCR® system (Thermo Fisher Scientific, Inc.). The primer pairs listed in Supplementary Table 1. Relative expression of each genes was normalized to *Gapdh* expression and estimated using the 2-ΔΔCq method [30]. Expression levels were presented as fold-change relative to the control.

2.9. Western blotting assay

Tissue lysates and whole cell lysates were extracted from gastrocnemius muscle tissues and cultured C2C12 myotubes, respectively, using RIPA lysis buffer (Sigma-Aldrich) supplemented with proteinase and phosphatase inhibitors (Thermo Fisher Scientific, Inc.). Protein concentrations were determined by Bradford colorimetric assay (Bio-Rad, Hercules, CA, USA). Total protein extracts (50μg) were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NuPAGE™ 4%–12%, Bis-Tris; Thermo Fisher Scientific, Inc.). Gels were transferred to membranes, saturated with blocking solution (5% milk and 0.1% Tween-20 in PBS), and incubated with primary antibodies overnight at 4 °C: myostatin (MSTN) (1:500; ab203076, Abcam), atrogin-1 (2 μg/ml; ab74023, Abcam), muscle RING-finger protein-1 (MuRF1, 1:1,000; ab172479, Abcam), myogenin (MyoG) (1:200; ab124800, Abcam), myoD1 (1:200; ab203383, Abcam), mitochondrial transcription factor A (mtTFA) (1:1,000; ab252432, Abcam), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) (1:1,000; PA5-72948, Thermo Fisher Scientific, Inc.), sirtuin1 (SIRT1) (1:1,000; ab189494, Abcam), general control nonderepressible 2 (GCN2) (1:1,000; ab302609, Abcam), p-GCN2 (Thr899, 1:1,000, ab75836, Abcam), eukaryotic initiation factor 2α (eIF2α) (1:1,000; 9722, Cell Signaling Technology), p-eIF2α (Ser51, 1:1,000; 9721, Cell Signaling Technology), activating transcriptional factor 4 (ATF4) (1:1,000; 11815, Cell Signaling Technology), protein kinase A catalytic-α (PKA C-α, 1:1,000; 4782, Cell Signaling Technology), p-PKA C-α (Thr197, 1:1,000, 5661, Cell Signaling Technology), nuclear factor-κB (NF-κB), p65 (1:1,000, 8242, Cell Signaling Technology), p-NF-κB p65 (Ser536, 1:1,000, 3033, Cell Signaling Technology), AKT (1:1,000; 4691, Cell Signaling Technology), p-AKT (Ser473, 1:1,000; 4060, Cell Signaling Technology), heat-shock transcription factor 1 (HSF-1, 1:1,000;

12972, Cell Signaling Technology), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1,000, 2118, Cell Signaling Technology).
The membranes were then washed thrice and incubated with goat anti-rabbit IgG H&L (HRP) (1:2,000 dilution; ab6721, Abcam) for 1 h at room temperature. The blots were developed with Clarity Western ECL Substrate (Bio-Rad) using iBright™ CL1500 Imaging System (Thermo Fisher Scientific, Inc.). Densitometric analysis was performed using ImageJ 64-bit Java 1.8.0. (National Institute of Health).

2.10. Measurement of cAMP in C2C12 myotubes

Intracellular cAMP concentrations in C2C12 myocytes were measured using Cyclic AMP XP® Assay kits (Cell Signaling Technology), according to the manufacturer's protocol.

2.11. Statistical analyses

Statistical analyses were performed using Prism, version 9 (GraphPad Software, San Diego, CA, USA). Data were analyzed using one-way ANOVA followed by Tukey's t-test as a post hoc test. P-values less than 0.05 were considered statistically significant. Data were presented as means ± standard deviation.

3. Results

3.1. Semaglutide prevents the physiological atrophy and hepatic injury in DDC-fed KK-Ay mice.

We initially evaluated the effects of semaglutide on physiological changes in the experimental groups. In ND-fed KK-A^y mice, no significant differences in body

length or weight were observed between the ND-Veh and ND-Sem groups, indicating a limited effect of semaglutide treatment for six weeks on physiological status (Figures 1A–1C). KK-A^{y} mice fed the DDC diet for three weeks had significantly lower body length and weight, with mice in the DDC-Veh group having approximately half the body weight of mice in ND-Veh group after six weeks of treatment (Figures 1A–1C). Interestingly, treatment with semaglutide attenuated DDC diet-induced physiological changes in KK-A^{y} mice (Figures 1A–1C). We next assessed glycemic status and hepatic phenotypes. Serum glucose levels in both ND-fed and DDC-fed KK-A^{y} mice confirmed the antidiabetic effect of semaglutide (Figure 1D). Treatment with semaglutide reduced serum levels of AST and ALT in both ND-fed and DDC-fed KK-A^{y} mice, whereas semaglutide had no effect on serum levels of albumin and total bilirubin (Figure 1E). Histological analyses demonstrated extensive hepatic steatosis in ND-fed KK-A^{y} mice (ND-Veh group), which was significantly attenuated by treatment with semaglutide in the ND-Sem group (Figure 1F). In accordance with the attenuating effects of semaglutide on hepatic steatosis, hepatic inflammation and CD45^{+} inflammatory cells were reduced in the ND-Sem group (Figure 1F–1H). In DDC-fed KK-A^{y} mice, significant levels of hepatic inflammation and fibrosis were observed in the DDC-Veh group, which was significantly attenuated in the DDC-Sem group (Figures 1F–1I). Along with the hepatic fibrosis development, hepatic and serum levels of IGF-1, known as a myotrophic factor, decreased in the DDC-Veh group compared to the ND-Veh group, and these levels notably increased in the DDC-Sem group (Figure 1J). As reported previously, the DDC diet-induced hepatic accumulation of MDA and 4-HNE, the final products of polyunsaturated fatty acid peroxidation (Figure 1K) [31]. Treatment with semaglutide significantly reduced hepatic levels of MDA and 4-HNE in DDC diet-fed KK-A^{y} mice (Figure 1K).

3.2. Attenuating effects of semaglutide on psoas muscle atrophy and decreases in grip strength in DDC-fed KK-A^{y} mice

Given the remarkable reductions in body length and weight in DDC diet-fed KK-A^{y} mice, we posited the presence of skeletal muscle atrophy in this model. Consequently, we chronologically assessed the CT-based measurement of psoas muscle area and the calculation of PMI (Figure 2A, B). PMI value was lower after three weeks of feeding in DDC-fed groups compared to ND-fed groups (Figure 2B). Of note, treatment with semaglutide ameliorated DDC-induced decreases in PMI values with significant difference at six weeks after the start of treatment (Figure 2B). Consistently with the reduced psoas muscle atrophy, treatment with semaglutide significantly attenuated DDC-induced reductions in gastrocnemius muscle weight (Figure 2C). We also evaluated muscular strength in mice by measuring hindlimb and forelimb grip strength. DDC-fed mice markedly lower both hindlimb and forelimb grip strength compared with ND-fed mice after three weeks of feeding (Figure 2D, E). Of note, treatment with semaglutide attenuated DDC-induced declines of hindlimb and forelimb grip strength at six weeks after the start of treatment (Figure 2D, E).

Moreover, we examined the effect of semaglutide on adipose tissue and leptin levels, the latter being one of the key regulators for skeletal muscle homeostasis. In ND-fed KK-A^{y} mice, treatment with semaglutide was found to reduce visceral fat volume estimated by CT images as well as plasma leptin level. In contrast, these parameters remained unchanged in DDC diet-fed KK-A^{y} mice (Supplementary Figure 1A and 1B). These findings suggest that the effect of semaglutide on adipose tissue was limited in the DDC-fed KK-A^{y} mice.

3.3. Semaglutide suppresses protein degradation and promotes myogenesis in the gastrocnemius muscle of DDC-fed KK-A^{y} mice

We next histologically assessed the diameter of gastrocnemius muscle fibers in mice. As shown in Figure 3A, H&E staining revealed atrophic changes in gastrocnemius muscle fibers in DDC-fed KK-A^{y} mice. Quantitative analysis demonstrated that the Feret diameter of gastrocnemius muscle fibers in the ND-Veh group had a normal distribution curve with a peak in the range of 41–45 μm, whereas the distribution of Feter diameters in the DDC-Veh group were obviously shifted to a smaller size with a peak in the range of 31–35 μm (Figure 3B). Compared to the DDC-Veh group, the Feret diameter in the DDC-Sem group had a broader distribution curve with a peak in the range of 35–40 μm, indicating the inhibitory effect of semaglutide on DDC-induced fiber atrophy in the gastrocnemius muscle (Figure 3B). Correspondingly, the average diameter of gastrocnemius muscle fibers was lower in the DDC-Veh group compared to the ND-Veh group, with this difference attenuated in the DDC-Sem group (Figure 3C). We next evaluated intramuscular protein levels of E3 ubiquitin ligases atrogin-1 and MuRF-1, crucial regulators of ubiquitin-mediated skeletal muscle protein degradation [15,32]. Intramuscular protein levels of Atrogin-1 and MuRF-1 were increased in the DDC-Veh group, with this difference significantly attenuated in the DDC-Sem group (Figure 3D). Intramuscular levels of MSTN, a myokine that inhibits muscle cell growth, was also markedly higher in the DDC-Veh group compared to the ND-Veh group, and this difference was attenuated in the DDC-Sem group (Figure 3D). In contrast, protein levels of myogenic factors, MyoD and MyoG, were in the DDC-Veh group, and this difference was attenuated in the DDC-Sem group (Figure 3E). In parallel with these changes in protein levels, we observed decreased mRNA expression levels of *Fbxo32*, *Trim63*, and *Mstn* and increased mRNA expression levels of

MyoD and *MyoG* in the DDC-Sem group compared to the DDC-Veh group (Figure 3F, G).

3.4. Semaglutide promotes mitochondrial biogenesis and inhibits inflammatory, oxidative stress, and integrated stress responses in the gastrocnemius muscle

We further aimed to elucidate the molecular mechanisms underlying the effects of semaglutide on skeletal muscle homeostasis. Mitochondrial dysfunction is known to trigger skeletal muscle atrophy [33]. We therefore evaluated the effects of semaglutide on mitochondrial biogenesis in the gastrocnemius muscle tissue. The DDC-Veh group had a marked decrease in intramuscular mRNA levels of genes related to mitochondrial biogenesis, including *Ppargc1a*, *Tfam*, and *Sirt1* (Figure 4A). These decreases in mitochondrial biogenesis-related gene expression were ameliorated in the DDC-Sem group (Figure 4A). Furthermore, semaglutide increased protein levels of PGC-1α, mtTFA, and SIRT1 in DDC-fed KK-A^{y} mice (Figure 4B). We next examined proinflammatory nuclear factor-kappa B (NF-κB) signaling in gastrocnemius muscle tissue. Intramuscular mRNA levels of proinflammatory cytokines including *Tnfa*, *Il6*, and *Il1b* were increased in the DDC-Veh group, and this difference was attenuated in the DDC-Sem group (Figure 4C). In parallel with the mRNA expression levels of proinflammatory cytokines, treatment with semaglutide reduced NF-κB p65 protein levels in the muscles of DDC-fed KK-A^{y} mice (Figure 4D).

DDC diet-induced skeletal muscle atrophy is reported to be mediated by the accumulation of oxidative stress [20]. We therefore assessed the effect of semaglutide on oxidative stress in the gastrocnemius muscle. Intramuscular levels of 4-HNE were increased in the DDC-Veh group compared to the ND-Veh

group, and this increase was attenuated in the DDC-Sem group (Figure 4E). Remarkably, treatment with semaglutide increased mRNA expression levels of antioxidant genes including *Hmox1*, *Nqo1*, and *Gstm3* in gastrocnemius muscle tissue from both ND-fed and DDC-fed KK-A^{y} mice (Figure 4F).

Moreover, it has been shown that chronic liver injury induces amino acid starvation to interfere with the synthesis of mammalian target of rapamycin (mTOR)-dependent skeletal muscle protein [34]. Our results showed that the GCN2/eIF2α/ATF4 pathway (a stress signaling pathway related to amino acid starvation) was activated, resulting in an increase in the mRNA level of *Sesn2*, which encodes Sestrin2 (a stress response protein), in the gastrocnemius muscle tissue of the DDC-Veh group (Figure 4G and 4H). Correspondingly to this signal transduction, the phosphorylation of p70S6K, a downstream target of mTOR, was repressed in the DDC-Veh group (Figure 4I). Of note, treatment with semaglutide significantly suppressed the activation of the GCN2/eIF2α/ATF4 signaling pathway and the upregulation of Sestrin2, and consequently recovered the phosphorylation of p70S6K (Figures 4G-4I).

3.5. Direct effects of semaglutide on palmitic acid-stimulated skeletal muscle cells

We next investigated the effects of semaglutide on GLP-1R activation in skeletal muscle cells. Immunofluorescent analysis was used to determine the expression of GLP-1R in skeletal muscle tissues from KK-A^{y} mice (Figure 5A). PA reportedly induces skeletal muscle cell injury via mitochondrial dysfunction and oxidative stress [35]. We therefore examined the effects of semaglutide on PA-stimulated mouse myocytes differentiated from C2C12 myoblasts *in vitro* (Figure 5B). GLP-1R was expressed in C2C12 myocytes, and the expression level of GLP-1R was not altered following treatment with PA or semaglutide

(Figure 5C). Stimulation with PA increased mRNA expression levels of *Fbxo32*, *Trim63*, and *Mstn* in a dose-dependent manner (Figure 5D), indicating increasing muscle protein degradation. PA at a concentration of 0.75 mM significantly increased skeletal protein degradation in C2C12 myocytes. Stimulation with PA also significantly reduced cell viability of C2C12 myocytes at concentrations over 0.75 mM (Supplementary Figure 2A). This dose was therefore used for all further experiments. We next examined the effect of semaglutide at varying doses (0.3, 0.6, and 0.9 μM) on mRNA expression levels of *Fbxo32*, *Trim63*, and *Mstn* following stimulation with PA. Semaglutide at a dose of 0.9 μM significantly decreased mRNA expression levels of *Fbxo32*, *Trim63*, and *Mstn* in PA-stimulated C2C12 myocytes (Figure 5E and Supplementary Figure 2B). We confirmed that 0.9 μM of semaglutide could efficiently suppress PA-stimulated reduction of C2C12 myocytes cell viability (Supplementary Figure 2C). Based on these results, semaglutide was used at a dose of 0.9 μM was used for all *in vitro* studies. PA decreased mRNA expression levels of *MyoD* and *MyoG*, with this difference attenuated by treatment with semaglutide (Figure 5F). Corresponding changes in protein levels were also observed (Figure 5G). These effects of semaglutide were negated by treatment with Ex-9, a selective GLP-1R antagonist, indicating that the myotrophic effects of semaglutide are mediated by GLP-1R signaling in skeletal muscle cells (Figure 5E–G).

3.6. Semaglutide promotes mitochondrial biogenesis and antioxidant activity via GLP-1R/cAMP signaling activation in skeletal muscle cells.

We next confirmed that intracellular cAMP production is induced by semaglutide-mediated GLP-1R activation in C2C12 myocytes. Semaglutide increased cAMP production by more than 50% compared to the vehicle, and

this effect of semaglutide was ameliorated in the presence of Ex-9, indicating that semaglutide acts via GLP-1R in C2C12 myocytes (Figure 6A). Increased cAMP production subsequently led to activation of two downstream pathways, PKA C-α and AKT, as indicated by the phosphorylation of PKA C-α (Thr197) and AKT (Ser473) (Figure 6B). As expected, activation of PKA signaling increased protein levels of HSF-1, while activation of AKT signaling inhibited the phosphorylation of NF-κB (Ser536) (Figure 6B).

Finally, we aimed to elucidate the functional mechanisms underlying the direct effects of semaglutide on PA-stimulated C2C12 myocytes. As shown in Figure 6C, stimulation with PA significantly decreased mRNA expression levels of mitochondrial biogenesis-related markers (*Ppargc1a*, *Tfam* and *Sirt1*), and this affect was attenuated by treatment with semaglutide. Semaglutide increased expression levels of antioxidant markers (*Hmox1*, *Nqo1*, and *Gstm3*) regardless of stimulation with PA (Figure 6D). These altered mRNA expression levels in response to treatment with semaglutide were abolished by Ex-9-mediated blockade of GLP-1R (Figures 6C and 6D). These results indicate that semaglutide increased mitochondrial biogenesis and antioxidant activity by directly activating GLP-1R in skeletal myocytes.

4. Discussion

In the present study, we assessed the inhibitory effect of semaglutide, an antidiabetic GLP-1RA, on skeletal muscle wasting developed by CLD under diabetic conditions. We employed the DDC-fed mouse model to create a model of CLD-associated skeletal muscle sarcopenia in rodents. Chronic feeding of the DDC diet was established as a model for cholestatic disease via formation of intraductal porphyrin plugs [36]. This model has also been used to analyze

the hepatic progenitor cell-derived liver regeneration in cases of chronic liver injury [37]. Recent studies have characterized the development of sarcopenia secondary to CLD in mice fed with the DDC diet [20]. In this model, decreased levels of myofibrillar proteins and activation of the ubiquitin-proteasome system are observed without impairment of caloric intake or immobility. Accordingly, DDC-induced accumulation of oxidative stress may be involved in skeletal muscle atrophy [20]. Moreover, we also approached to elucidate the impact of the semaglutide-mediated antidiabetic effect on CLD-related skeletal muscle atrophy. To this end, we chose KK-A^y mice that spontaneously develop hyperglycemia, hyperinsulinemia, glucose intolerance, and obesity as well as mild hepatic steatosis by eight weeks of age.

Multiple signaling pathways were shown to mediate the suppressive effects of semaglutide on skeletal muscle atrophy in the DDC-fed KK-A^y diabetic mice (Figure 6E). First, the improvement of hepatic function observed upon treatment with semaglutide might indicate its anti-sarcopenic effect. Semaglutide attenuated hepatic dysfunction and suppressed the decline of hepatic and serum levels of IGF-1 in DDC-fed KK-A^y mice. IGF-1 plays a pivotal role in muscle mass integrity, strength development, and degeneration, and also increases the proliferative capacity of muscle satellite cells, whereas the impaired liver function decreases hepatic production and secretion of IGF-1 [38,39]. We suggested that semaglutide could suppress the decline in IGF-1 levels by attenuating hepatic dysfunction and may contribute to the inhibition of skeletal muscle atrophy.

Moreover, our findings revealed that semaglutide suppressed hepatic ROS levels. In DDC-fed KK-A^y mice, hepatic ROS appears to accumulate due to hyperglycemia and DDC-mediated hepatotoxicity, two pathological factors that contribute to hepatic injury [40]. Several studies have reported that GLP-1RAs

decrease hepatic accumulation of ROS in rodent models of obesity and type 2 diabetes [41,42]. In accordance with these reports, treatment with semaglutide was found to decrease the intramuscular levels of proinflammatory cytokines and 4-HNE along with a concurrent improvement of hepatic pathological changes and ROS accumulation. Moreover, semaglutide also inhibited the integrated stress response in DDC-fed KK-A^{y} mice. Chronic liver injury has been known to cause amino acid starvation and activate the GCN2/eIF2α/ATF4 pathway and Sestrin2 expression, negatively regulating mTORC1-mediated skeletal muscle protein synthesis [34]. Our findings showed that semaglutide suppressed this signal transduction resulting in the recovery of mTOR activity in DDC-diet fed KK-A^{y} mice. We speculate that semaglutide could suppress amino acid starvation by improving hepatic inflammation and fibrosis; however, further investigations are required to elucidate alterations in the amino acid status of the present model.

Second, semaglutide directly affected myocytes via activation of GLP-1R, stimulating myogenesis and suppressing UPS-mediated muscle degradation. We observed no significant differences in the GLP-1R levels of muscle tissues between the experimental groups in the present study, which was in agreement with the results of the *in vitro* assay demonstrating that GLP-1R expression remained unchanged following stimulation with PA or treatment with semaglutide in C2C12 myocytes. In addition to increasing the expression of myogenic markers in PA-stimulated C2C12 myocytes, treatment with semaglutide decreased the expression of UPS markers and MSTN. Recent studies have revealed the functional role of GLP-1R in maintaining homeostasis in skeletal muscle cells. *Silveira* et al. demonstrated that cAMP/PKA C-α signaling inhibits UPS-mediated protein degradation, while AKT signaling augments protein synthesis in the skeletal muscle [43]. PKA C-α is reported to

activate HSF-1, which transcriptionally upregulates heat-shock protein 70 (HSP70) expression [44]. HSP70 protects against muscle injury, promotes muscle regeneration and recovery, and maintains skeletal muscle mass and integrity [45]. Moreover, activation of AKT signaling can directly inhibit NF-κB phosphorylation, which may in turn interfere with the promoter regions of MSTN [46]. AKT signaling has also been shown to stimulate myogenic differentiation by upregulating myogenic factor expression, which results in the promotion of skeletal myogenesis [47–49]. The results of our *in vitro* study consistently demonstrated that semaglutide increased cAMP production, activated PKA/HSF-1 signaling, and inhibited NF-κB phosphorylation through AKT signaling in PA-stimulated C2C12 myocytes. Of note, these effects were abolished after treatment with Ex-9, a GLP-1R antagonist, indicating that semaglutide acts by directly stimulating GLP-1R in myocytes.

Moreover, GLP-1R agonists have been reported to modulate mitochondrial function in various cell types [50–52]. Notably, Khin et al. reported that dulaglutide enhanced mitochondrial biogenesis and function by suppressing NF-kB signaling in the skeletal muscle of elder mice [14]. Although mitochondrial biogenesis-related markers were downregulated in DDC-fed diabetic mice in the current study, treatment with semaglutide significantly increased the expression of these markers in the gastrocnemius muscle tissue. Similarly, Kamiya et al. reported that agonistic stimulation of GLP-1R reduced ROS accumulation by activating the nuclear factor erythroid 2-related factor 2 (NRF2) pathway in skeletal muscle cells [53]. Our results also demonstrated that treatment with semaglutide decreased 4-HNE levels and increased expression of the NRF2-regulated antioxidant genes *Hmox1*, *Nqo1*, and *Gstm3* in the gastrocnemius muscle tissue. Particularly, these semaglutide-mediated effects were observed in PA-stimulated C2C12 myocytes, which were abolished

by Ex-9. These findings indicate that the effects of semaglutide on mitochondrial biogenesis and accumulation of ROS are mediated by GLP-1R stimulation in skeletal muscle cells.

The empirical results reported in this study should be considered in light of some limitations. First, we did not fully elucidate the effects of semaglutide on adipose tissue. Our results found that treatment with semaglutide did not change CT image-estimated visceral fat volume and serum leptin levels in DDC-fed KK-A^{y} mice. Thus, we speculate that the effects of semaglutide on the adipose tissue and leptin might not contribute substantially to the anti-sarcopenic effect in this model. Meanwhile, it has been reported that white adipose tissue also plays a key role in regulating muscle mass, especially leptin secretion by adipose tissue [54]. To clarify the relevance of lipid metabolism to the anti-sarcopenic effect in detail, we need to analyze histological changes and molecular alterations in adipocytes in the model used in this study and in other models. Second, the changes in serum ammonia levels were obscure, although semaglutide reduced intramuscular expression of myostatin, a key factor in CLD-related sarcopenia. Qiu et al. demonstrated that hyperammonemia increased the expression of myostatin by activating NF-κB signaling [55]. These findings indicate that semaglutide may improve hyperammonemia and hepatic pathological phenotypes in DDC-fed KK-A^{y} mice.

The results of the present study demonstrate that semaglutide attenuates CLD-related skeletal muscle atrophy in DDC-fed diabetic mice. The effects of semaglutide in this model included: i) protective effects on hepatic inflammation, fibrosis, and ROS accumulation, and ii) activation of GLP-1R signaling activation leading to inhibition of UPS-mediated proteolysis and promotion of myogenesis in myocytes. Of note, semaglutide is clinically available and no hepatic or renal toxicity was observed in the present study. Accordingly,

semaglutide may have potential as a novel therapeutic option for treating sarcopenia in cirrhotic patients. Further studies are required to translate the findings of the present study into clinical applications.

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Figure legends

Figure 1. Effects of semaglutide on hepatic phenotypes in DDC-fed KK-A^{y} mice. (A) Representative whole-body photographs of experimental mice. (B and C) Changes in the body lengths and body weights of the mice during the experimental period. The comparison was performed between the groups at the last time point. (D and E) Serum levels of glucose, aspartate transaminase (AST), alanine aminotransferase (ALT), albumin (Alb) and total bilirubin (T-bil) at 6 weeks of treatment. (F) Representative microphotographs of liver sections stained with hematoxylin and eosin (H&E), CD45 and Sirius-Red in the experimental groups. Scale bar, 50 μm. pv, portal vein. (G and H) Semi-quantification of (G) inflammation cells (%) and (H) CD45^{+} inflammatory cells/DAPI^{+} cells. (I) Semi-quantitation of Sirius-Red-stained fibrosis by NIH imageJ software at a 400-fold magnification. Quantitative values are indicated as fold changes to the values of ND-Veh group. (J) Hepatic (left) and serum (right) concentrations of insulin-like growth factor 1 (IGF-1) at 6 weeks of treatment. (K) Hepatic levels of malondialdehyde (MDA) (left) and 4-hydoxy-2-nonenal (4-HNE) (right) at 6 weeks of treatment. Data are the mean ± SD (n=10). Counted images are representative of five independent experiments for each mouse (G-I). *P<0.05 and **P<0.01, significant difference between groups. N.S, not significant; ND, normal diet-fed group; DDC, diethoxycarbonyl-1,4-dihydrocollidine diet-fed group; Veh, vehicle-treated group; Sem, semagutide-treated group.

Figure 2. Effects of semaglutide on skeletal muscle mass and strength in DDC-fed KK-A^{y} mice. (A) Representative images on a single CT slice at the level of L3 pedicle at 6 weeks of treatment. (B) Psoas muscle mass index (PMI;

cross sectional area/height^2) was assessed on a single CT slice at the level of L3 pedicle with image analysis system (left panel). Chronological changes in the calculated PMI during experimental period (middle panel). PMI at 6 weeks of treatment (right panel). (C) Ratio of gastrocnemius muscle weight to body weight at 6 weeks of treatment. (D) Chronological changes in the hindlimb grip strength during the experimental period (left panel). Hindlimb grip strength at 6 weeks of treatment (right panel). (E) Chronological changes in the forelimb grip strength during the experimental period (left panel). Forelimb grip strength at 6 weeks of treatment (right panel). Data are the mean ± SD (n=10). *P<0.05 and **P<0.01, significant difference between groups. N.S, not significant; ND, normal diet-fed group; DDC, diethoxycarbonyl-1,4-dihydrocollidine diet-fed group; Veh, vehicle-treated group; Sem, semagutide-treated group.

Figure 3. Effects of semaglutide on protein degradation and myogenesis of gastrocnemius muscle in DDC-fed KK-A^y mice. (A) Representative microphotographs of gastrocnemius muscle sections stained with H&E in the experimental groups. Scale bar: 25 μm. (B) Minimal Feret diameters were determined in gastrocnemius muscle cross-sections. Fiber diameters were grouped from 0 to 75 μm and values expressed as the percentage of the total fibers quantified. (C) Average value of minimal Feret diameters of gastrocnemius muscle fiber. (D and E) Western blots for (D) Atrogin-1, MuRF-1 and MSTN (Myostatin), and (E) MyoD and MyoG (Myogenin) in gastrocnemius muscle tissues. GAPDH was used as the loading control for western blot analysis. (F and G) Relative mRNA expression levels of (F) *Fbxo32*, *Trim63* and *Mstn*, and (G) *MyoD* and *MyoG* in the gastrocnemius muscle of experimental mice. *Gapdh* was used as an internal control for qRT-PCR. Counted images are representative of five independent experiments for each mouse (B and C).

Quantitative values are indicated as fold changes to the values of ND-Veh group. Data are the mean ± SD (n=10). *P<0.05 and **P<0.01, significant difference between groups. H&E, hematoxylin and eosin; ND, normal diet-fed group; DDC, diethoxycarbonyl-1,4-dihydrocollidine diet-fed group; Veh, vehicle-treated group; Sem, semagutide-treated group; MuRF-1, muscle RING-finger protein-1; *Fbxo32*, F-Box protein 32; *Trim63*, Tripartite Motif Containing 63; *Mstn*, myostatin.

Figure 4. Effects of semaglutide on mitochondrial biogenesis, inflammatory, oxidative stress and integrated stress response of gastrocnemius muscle in DDC-fed KK-A^{y} mice.

(A, C, F and H) Relative mRNA expression levels of (A) *Ppargc1a*, *Tfam* and *Sirt1*, (C) *Tnfa*, *Il6* and *Il1b*, (F) *Hmox1*, *Nqo1* and *Gstm3*, and (H) *Sesn2* in the gastrocnemius muscle of experimental mice. *Gapdh* was used as an internal control for qRT-PCR. Quantitative values are indicated as fold changes to the values of ND-Veh group. (B, D and G) Western blots for (B) PGC-1α, mtTFA, and SIRT1 (D) NF-κB p65, and (G) p-GCN2, t-GCN2, p-eIF2α, t-eIF2α and ATF4 in gastrocnemius muscle tissues. GAPDH was used as the loading control for western blot analysis. (E) Muscular levels of 4-hydoxy-2-nonenal (4-HNE) at 6 weeks of treatment. (I) The ratio of p-p70S6K$^{(Thr389)}$/total p70S6K levels gastrocnemius muscle tissues. Quantitative values are indicated as fold changes to the values of ND-Veh group.

Data are the mean ± SD (n=10). *P<0.05 and **P<0.01, significant difference between groups. ND, normal diet-fed group; DDC, diethoxycarbonyl-1,4-dihydrocollidine diet-fed group; Veh, vehicle-treated group; Sem, semagutide-treated group; *Ppargc1a*, peroxisome proliferator-activated receptor γ coactivator-1α; *Tfam*, mitochondrial transcription factor A; *Sirt1*, sirtuin1; *Tnfa*,

tumor necrosis factor α; *Hmox1*, heme oxygenase 1; *Nqo1*, NAD(P)H quinone dehydrogenase 1; *Gstm3*, glutathione S-transferase Mu 3; GCN2, general control nonderepressible 2; eIF2α, eukaryotic initiation factor 2α; ATF4, ctivating transcriptional factor 4; *Sesn2*, Sestrin 2; p70S6K, p70 ribosomal protein S6 kinase β-1

Figure 5. Direct effect of semaglutide on C2C12 myocytes through GLP-1R. (A) Representative microphotographs of immunofluorescence with GLP-1R/DAPI staining in the gastrocnemius muscle sections of experimental groups. Scale bar: 25 μm. (B) In vitro experimental protocol. (C and G) Western blots for (C) GLP-1R, and (G) Atrogin-1, MuRF-1, MSTN, MyoD and MyoG in the palmitic acid (PA)-stimulated mouse C2C12 myocytes. GAPDH was used as the loading control for western blot analysis. (D) Dose-dependent effects of PA (0-1 mM) on the mRNA expression levels of *Fbxo32*, *Trim63* and *Mstn* in C2C12 myocytes. (E and F) Relative mRNA expression levels of (E) *Fbxo32*, *Trim63* and *Mstn*, and (F) *MyoD* and *MyoG* in the PA-stimulated C2C12 myocytes. (C, E, F, G) C2C12 myocytes were treated with PA (0.75 mM) and/or Sem (0.9 μM) and EX-9 (0.2 μM). *Gapdh* was used as an internal control for qRT-PCR. Quantitative values are indicated as fold changes to the values of DMSO+veh group. Data are the mean ± SD (n=10). *P<0.05 and **P<0.01, significant difference between groups. ND, normal diet-fed group; DDC, diethoxycarbonyl-1,4-dihydrocollidine diet-fed group; Veh, vehicle-treated group; Sem, semagutide-treated group; *Fbxo32*, F-Box protein 32; *Trim63*, Tripartite Motif Containing 63; *Mstn*, myostatin; MuRF-1, muscle RING-finger protein-1; Ex-9, exendin(9-39).

Figure 6. Molecular changes in semaglutide-mediated C2C12 myocytes.

(A) Quantification of cAMP levels in the palmitic acid (PA)-stimulated C2C12 myocytes. (B) Western blots for PKA C-α phosphorylation, HSF-1, AKT phosphorylation and NF-κB p65 phosphorylation in the PA-stimulated C2C12 myocytes. GAPDH was used as the loading control for western blot analysis. (C and D) Relative mRNA expressions of (C) *Ppargc1a*, *Tfam* and *Sirt1*, and (D) *Hmox1*, *Nqo1* and *Gstm3* in the PA-stimulated C2C12 myocytes. *Gapdh* was used as an internal control for qRT-PCR. (E) Graphic representation of the effects of semaglutide on chronic liver disease-related skeletal muscle atrophy in diabetic mice. (A−D) C2C12 myocytes were treated with PA (0.75 mM) and/or Sem (0.9 μM) and EX-9 (0.2 μM). Quantitative values are indicated as fold changes to the values of DMSO+veh group (A, C and D). Data are the mean ± SD (n=10). *P<0.05 and **P<0.01, significant difference between groups. DDC, diethoxycarbonyl-1,4-dihydrocollidine diet; Sem, semagutide; Ex-9, exendin(9-39); *Ppargc1a*, peroxisome proliferator-activated receptor γ coactivator-1α; *Tfam*, mitochondrial transcription factor A; *Sirt1*, sirtuin1; 4-HNE, 4-hydoxy-2-nonenal; *Hmox1*, Heme Oxygenase 1; *Nqo1*, NAD(P)H quinone dehydrogenase 1; *Gstm3*, Glutathione S-Transferase Mu 3; ROS, reactive oxygen species.