Note

***Lactobacillus curvatus* CP2998 Prevents Dexamethasone-Induced Muscle Atrophy in C2C12 Myotubes**

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Summary To investigate whether heat-killed *Lactobacillus curvatus* CP2998 (CP2998) inhibits glucocorticoid-induced myotube atrophy which is associated with the ubiquitin-proteasome system, mouse skeletal muscle C2C12 myotubes were treated with dexamethasone (DEX) in the presence or absence of CP2998. DEX exposure significantly decreased myotube diameters and increased mRNA expression levels of *MuRF1* and *MAFbx*, E3 ubiquitin ligases. CP2998 treatment restored myotube diameters and dose dependently decreased mRNA expression levels of these E3 ubiquitin ligases. CP2998 treatment also inhibited DEX-induced glucocorticoid dependent transcription. Our results suggest that CP2998 prevents DEX-induced muscle atrophy by suppressing glucocorticoid receptor activation.

Key Words lactic acid bacteria, sarcopenia, skeletal muscle, glucocorticoid, FoxO, KLF15, *MuRF1*, *MAFbx*, ubiquitin ligases

Skeletal muscle is the largest organ and major protein reserve in the human body. It supports and moves the body, metabolizes energy substrates, and regulates body temperature. Muscle mass maintenance is dependent on the balance between synthesis and breakdown of myofibrillar proteins (1). An age-related decline in muscle mass and strength, known as sarcopenia, is often an important antecedent to the onset of disability in older adulthood (2–4). Although the precise mechanism of sarcopenia remains unknown, hypersecretion of glucocorticoids may contribute to the muscle catabolism that occurs during the aging process (4).

Glucocorticoids activate the ubiquitin-proteasome system via glucocorticoid receptor (GR) activation. Glucocorticoids induce the expression of forkhead box O 1/3 (FoxO1/3) and kruppel like factor 15 (KLF15) proteins (5). Activated GR, FoxO1/3 and KLF15 upregulate the expression of genes encoding E3 ubiquitin ligases, such as muscle RING finger 1 (*MuRF1*) and muscle atrophy F-box (*MAFbx*) (5–8). Overexpression of *MAFbx* in myotubes produces atrophy, whereas mice deficient in *MuRF1* and *MAFbx* are resistant to atrophy (9). Glucocorticoids accelerate muscle atrophy by upregulating E3 ubiquitin ligases.

Lactic acid bacteria (LAB) are the microorganisms

most frequently used as probiotic agents. LAB improve gut health (10), enhance immunity (11) and alter stress sensitivity (12). Furthermore, LAB reduce inflammation, an effect correlated with the improvement in skeletal muscle atrophy markers (13). LAB are an attractive strategy for sarcopenia treatment because many products containing LAB are sold worldwide (14).

We selected *Lactobacillus curvatus* CP2998 (CP2998) as a commercial starter culture and examined whether heat-killed CP2998 suppressed dexamethasone (DEX; synthetic glucocorticoid)-induced muscle atrophy in C2C12 myotubes.

Materials and Methods

Preparation of experimental samples. CP2998 was isolated from fermented food and stored in the culture collection library of Asahi Group Holdings, Ltd. Bacterial cells were cultured for 28 h at 30°C in Lactobacilli MRS broth (Becton, Dickinson and Company, NJ, USA). The bacterial cells were harvested by centrifugation, washed with water, killed by heating at 90°C for 30 min and lyophilized.

Cell culture and experimental treatments. C2C12 myoblasts (DS Pharma Biomedical, Osaka, Japan) were seeded into 12-well plates and cultured in DMEM containing 4.5 g/L glucose (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10%

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FBS (Sigma-Aldrich, SLE, USA) plus antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin; Nacalai Tesque, Inc., Kyoto, Japan) at 37°C in a humidified 5% CO₂ atmosphere. At 90% confluence, the cells were differentiated into myotubes in DMEM containing 4.5 g/L glucose plus 2% horse serum (Invitrogen, CA, USA) and antibiotics for 6 d. C2C12 myotubes were treated with 1 μ M DEX (FUJIFILM Wako Pure Chemical Corporation) and heat-killed CP2998 cells for 24 h.

Measurement of myotube diameter. After washing with PBS, differentiated myotubes were photographed at $\times 40$ magnification using the CKX53 microscope (OLYMPUS, Tokyo, Japan). For each group, the diameters of 100 myotubes were measured using the high-power field with Image J.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from C2C12 myotubes using an RNeasy Plus Mini Kit (QIAGEN, Venlo, Netherlands). High Capacity cDNA Reverse Transcription (random primer) Kit (Applied Biosystems, CA, USA) was used to prepare cDNA. qRT-PCR was conducted using the Fast SYBR Green Master Mix (Applied Biosystems). Oligonucleotide primers are shown in Table 1. Primer sequences were designed as previously reported (15) or using Primer3 software (16, 17), and synthesized by Sigma-Aldrich. GAPDH mRNA was used as the normalization control for each sample, and mRNA expression levels were analyzed for fold change ($\Delta\Delta$ Ct). The data were representa-

tive of three independent experiments.

Luciferase assay. C2C12 myoblasts were seeded into 24-well plates 24 h before the transfection with 400 ng/well glucocorticoid response element luciferase (GRE-Luc) vector (Panomics, CA, USA) using PolyFect reagent (QIAGEN). Twenty four hours after transfection, 100 μ g/mL of heat-killed CP2998 cells or 10 μ M RU486 (Sigma-Aldrich), the GR antagonist, were added. The C2C12 cells were then cultured for 2 h before 1 μ M of DEX was added. Twelve hours after DEX treatment, the cells were washed with PBS and harvested into 100 μ L of passive lysis buffer (Promega, WI, USA).

Table 1. qRT-PCR primer sequences used in this study.

Gene	Sequence (5'-3')	Reference or source
<i>MuRF1</i>	F: TGTCTGGAGGTCGTTTCCG R: ATGCCGGTCCATGATCACTT	15) 15)
<i>MAFbx</i>	F: ATCCCAGCACACGACAACAC R: CGGCAACTGCATCTCTTCC	This study This study
<i>FoxO3</i>	F: TCGTCTCTGAACCTCTGCGT R: TGGAGTGTCTGGTTGCCGT	15) 15)
<i>KLF15</i>	F: CTGCAGCAAGATGTACACCAA R: GCCTTGACAACATCTGAGC	This study This study
<i>GAPDH</i>	F: ATGGCCTTCCGTGTTCTCTAC R: TGCCTGCTTCACCACCTTC	This study This study

Fig. 1. Effects of CP2998 on DEX-induced muscle atrophy. (A) The diameters of C2C12 myotubes incubated with 100 μ g/mL CP2998. The expression levels of *MuRF1* (B), *MAFbx* (C), *FoxO3* (D), and *KLF15* (E). (F) GR activity incubated with 100 μ g/mL CP2998 or 10 μ M RU486. Error bars show SD (A: $n = 100$, B–E: $n = 6$). Different letters represent significant differences at $p < 0.05$ (Tukey-Kramer test).

Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega) using the Turner Designs Luminometer Model TD-20/20. Renilla luciferase plasmid was co-transfected to normalize transfection assays. The data were representative of three independent experiments.

Statistical analysis. Data analysis was performed using the Tukey-Kramer test and BellCurve for Excel version 2.02 software (SSRI, Tokyo, Japan). Values of $p < 0.05$ were considered statistically significant. Graphs represent means $\pm$ standard deviations (SD), as specified in each figure legend.

Results and Discussion

We investigated the effects of CP2998 on DEX-induced muscle atrophy by treating myotubes with DEX ($1 \mu\text{M}$) in the presence or absence of CP2998 (10, 100 and $1,000 \mu\text{g/mL}$) for 24 h. DEX treatment decreased the diameters of the myotubes (Fig. 1A) while CP2998 treatment prevented the DEX-induced reduction in myotube diameter. The results suggest that heat-killed CP2998 prevents DEX-induced muscle atrophy in C2C12 myotubes.

To examine the molecular mechanisms of the suppression of DEX-induced muscle atrophy by CP2998, we measured mRNA expression levels of genes involved in the ubiquitin-proteasome system. DEX treatment induced the expression of *MuRF1*, *MAFbx*, *FoxO3* and *KLF15*, which were all suppressed by treatment with CP2998 in a dose dependent manner (Fig. 1B–E).

Leucine (18), pyroglutamate (19), sulforaphane (20) and other materials (21–23) had similar effects to CP2998; however, to our knowledge, this is the first study to report on the inhibiting effects of LAB on muscle atrophy.

Finally, we examined the effect of CP2998 on GR-dependent transcription activity in C2C12 myoblasts using a GRE-Luc reporter assay. DEX treatment activated GR-dependent transcription (Fig. 1F), while the presence of CP2998 or RU486 (GR antagonist) inhibited the transactivation. The data implies that CP2998 prevents DEX-induced muscle atrophy by suppressing GR activation.

Previous studies have proven that LAB prevents muscle atrophy in vivo in other organs: *Lactobacillus reuteri* 100–23 and *Lactobacillus gasseri* 311476 reduced leukemia-associated inflammation and related disorders in the muscle (13). Changes in gut microbiota have been reported to regulate muscle mass (24). In addition, heat-killed *Enterococcus faecium* R30 increased the velocity of red blood cells, blood flow and capillaries in the soleus muscle via muscle sympathetic nerve activity (25). Increased blood flow caused angiogenesis and suppressed capillary regression (26). Our results differ from previous reports because we show that CP2998 directly suppresses DEX-induced muscle atrophy. Further studies are needed to reveal the exact mechanism of CP2998 action in vivo.

The effects of *Lactobacillus* species vary by strain, such as improving gut health (10), enhancing immunity (11)

and altering stress sensitivity (12). CP2998 is suggested to be an attractive strategy for sarcopenia treatment because various products containing LAB are already popular worldwide (15). In conclusion, our data indicate that CP2998 prevents DEX-induced muscle atrophy via suppression of GR-dependent transcription. These findings suggest that CP2998 could be a new therapeutic application in sarcopenia.

Disclosure of state of COI

No conflicts of interest to be declared.

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