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

Ryo Katsuki1, Shinji Sakata1, Reiko Nakao2, Katsutaka Oishi2,3,4,5 and Yasunori Nakamura1

1 Department of Lactic Acid Bacteria Technology Core Technology Laboratories, Asahi Quality & Innovations, Ltd., Moriya, Ibaraki 302–0106, Japan
2 Biological Clock Research Group, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305–8566, Japan
3 Department of Applied Biological Science, Graduate School of Science and Technology, Tokyo University of Science, Noda, Chiba 278–8510, Japan
4 Department of Computational and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba 277–0882, Japan
5 School of Integrative and Global Majors (SIGMA), University of Tsukuba, Tsukuba, Ibaraki 305–8577, Japan

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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 O1/3 (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%
FBS (Sigma-Aldrich, SLE, USA) plus antibiotics (100 U/mL penicillin, 100 μg/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 μM 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 ×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 (ΔΔCt). The data were representative 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 μg/mL of heat-killed CP2998 cells or 10 μM RU486 (Sigma-Aldrich), the GR antagonist, were added. The C2C12 cells were then cultured for 2 h before 1 μM of DEX was added. Twelve hours after DEX treatment, the cells were washed with PBS and harvested into 100 μL of passive lysis buffer (Promega, WI, USA).

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

| Gene   | Sequence (5′-3′) | Reference or source |
|--------|-----------------|---------------------|
| MuRF1  | F: TGCTCTGGAAGTGTTTCCG | 15)                 |
|        | R: ATGCCGGTGCTGATCATGACTT | 15)                 |
| MAFbx  | F: ATCCCACACAGCACACACAC | This study          |
|        | R: CGGAAACTGCACTCTTCCC | This study          |
| FoxO3  | F: TCCTCTCGAATCTCTTCCGCTG | 15)                 |
|        | R: TGGAGTGTCCTGTCGTTCCCGT | 15)                 |
| KLF15  | F: CTGCAGCAAAGATGTCACACA | This study          |
|        | R: GCCGTGACAATCTGACAGC | This study          |
| GAPDH  | F: ATGGCCTCTCGTGCTGTCTAC | This study          |
|        | R: TGCCCTGTCACCACCTTC | This study          |

Fig. 1. Effects of CP2998 on DEX-induced muscle atrophy. (A) The diameters of C2C12 myotubes incubated with 100 μg/mL CP2998. The expression levels of MuRF1 (B), MAFbx (C), FoxO3 (D), and KLF15 (E). (F) GR activity incubated with 100 μg/mL CP2998 or 10 μM 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±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 $\mu$m) in the presence or absence of CP2998 (10, 100 and 1,000 $\mu$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), pyrophia yezosens peptide (19), sulfurophane (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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REFERENCES

1) Hoffman EP, Nader GA. 2004. Balancing muscle hypertrophy and atrophy. Nat Med 10: 584–585.
2) Srikantham P, Karlamangla AS. 2014. Muscle mass index as a predictor of longevity in older adults. Am J Med 127: 547–553.
3) Reubornoff R. 2001. Origins and clinical relevance of sarcopenia. Can J Appl Physiol 26: 78–89.
4) Sakuma K, Yamaguchi A. 2012. Sarcopenia and age-related endocrine function. Int J Endocrinol 2012: 127362.
5) Shimizu N, Yoshikawa N, Ito N, Maruyama T, Suzuki Y, Takeda S, Nakue J, Tagata Y, Nishitani S, Takehana K, Sano M, Fukuda K, Suematsu M, Morimoto C, Tanaka H. 2011. Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. Cell Metab 13: 170–182.
6) Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, Goldberg AL. 2004. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell 117: 399–412.
7) Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, Glass DJ. 2004. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell 14: 395–403.
8) Waddell DS, Baehr LM, van den Brandt J, Johnsen SA, Reichardt HM, Furlow JD, Bodine SC. 2008. The glucocorticoid receptor and FOXO1 synergistically activate the skeletal muscle atrophy-associated MuRF1 gene. Am J Physiol Endocrinol Metab 295: E785–E797.
9) Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan QZ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ. 2001. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294: 1704–1708.
10) Venturi A, Gionchetti P, Rizzello F, Johansson R, Zucconi E, Brigidi P, Matteuzzi D, Campieri M. 1999. Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. Aliment Pharmacol Ther 13: 1103–1108.
11) Gill HS, Rutherford KJ, Prasad J, Gopal PK. 2000. Enhancement of natural and acquired immunity by Lacto-
tobacillus rhamnosus (HN001), Lactobacillus acidophilus (HN017) and Bifidobacterium lactis (HN019). Br J Nutr 83: 167–176.

12) De Palma G, Collins SM, Bercik P, Verdu EE. 2014. The microbiota-gut-brain axis in gastrointestinal disorders: stressed bugs, stressed brain or both? J Physiol 592: 2989–2987.

13) Bindels LB, Beck R, Schakman O, Martin JC, De Backer F, Sohet FM, Dewulf EM, Pachikian BD, Neyrinck AM, Thissen JP, Verrax J, Calderon PB, Pot B, Grangette C, Cani PD, Delzenne NM. 2012. Restoring specific lactobacilli levels decreases inflammation and muscle atrophy markers in an acute leukemia mouse model. PLoS One 7: e37971.

14) Saxelin M, Chuang NH, Chassy B, Rautelin H, Makela PH, Salminen S, Gorbach SL. 1996. Lactobacilli and bacteremia in southern Finland, 1989–1992. Clin Infect Dis 22: 564–566.

15) Gilson H, Schakman O, Combaret L, Lause P, Grobet L, Attiax D, Ketelslegers JM, Thissen JP. 2007. Myostatin gene deletion prevents glucocorticoid-induced muscle atrophy. Endocrinology 148: 452–460.

16) Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res 40: e115.

17) Koressaar T, Remm M. 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23: 1289–1291.

18) Wang XJ, Yang X, Wang RX, Jiao HC, Zhao JP, Song ZG, Lin H. 2012. Leucine alleviates dexamethasone-induced suppression of muscle protein synthesis via synergy involvement of mTOR and AMPK pathways. Biosci Rep 36: e00346.

19) Lee MK, Kim YM, Kim IH, Choi YH, Nam TJ. 2017. Pyrobutyric acid produces the down-regulation of MuRF1 in mouse C2C12 myotubes. Mol Med Rep 15: 3507–3514.

20) Son YH, Jung EJ, Kim YW, Lee JH. 2017. Sulfuraphane prevents dexamethasone-induced muscle atrophy via regulation of the Akt/mTOR axis in C2C12 myotubes. Biomed Pharmacother 95: 1486–1492.

21) Kim H, Jung M, Park R, Jo D, Choi I, Choe J, Oh WK, Park J. 2018. Conessine treatment reduces dexamethasone-induced muscle atrophy by regulating MuRF1 and atrogin-1 expression. J Microbiol Biotechnol 28: 520–526.

22) Sun LJ, Sun YN, Chen SJ, Liu S, Jiang GR. 2017. Resveratrol attenuates skeletal muscle atrophy induced by chronic kidney disease via MuRF1 signaling pathway. Biochem Biophys Res Commun 487: 83–89.

23) Li F, Li X, Peng X, Sun L, Jia S, Wang P, Ma S, Zhao H, Yu Q, Huo H. 2017. Ginsenoside Rg1 prevents starvation-induced muscle protein degradation via regulation of AKT/mTOR/FoxO signaling in C2C12 myotubes. Exp Ther Med 14: 1241–1247.

24) Everard A, Lazarevic V, Derrien M, Girard M, Muccioli GG, Muccioli GM, Neyrinck AM, Possemiers S, Van Holle A, François P, de Vos WM, Delzenne NM, Schrenzel J, Cani PD. 2011. Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. Diabetes 60: 2775–2786.

25) Hirayama Y, Nakanishi R, Tatagaki A, Maeshige N, Kondo H, Ishihara A, Roy RR, Fujino H. 2017. Enterococcus faecium strain R30 increases red blood cell velocity and prevents capillary regression in the soleus of hindlimb-unloaded rats via the eNOS/VEGF pathway. Microcirculation 24.

26) Tamida M, Shen J, Niijima A, Yamatodani A, Oishi K, Ishida N, Nagai K. 2008. Effects of olfactory stimulations with scents of grapefruit and lavender oils on renal sympathetic nerve and blood pressure in Clock mutant mice. Auton Neurosci 139: 1–8.