Inhibitory Effect of the Glycerophosphate Moiety of Lipoteichoic Acid from Lactic Acid Bacteria on Dexamethasone-Induced Atrogin-1 Expression in C2C12 Myotubes

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Summary Atrogin-1, which is an important regulator of ubiquitin-mediated protein degradation in skeletal muscle, is a major marker of muscle loss and disuse muscle atrophy. To investigate which components of lactic acid bacteria (LAB) suppress dexamethasone (DEX)-induced atrogin-1 expression, mouse skeletal muscle C2C12 myotubes were treated with DEX in the presence or absence of components of LAB. Heat-killed cells and lipoteichoic acid (LTA) derived from five LAB strains significantly suppressed DEX-induced atrogin-1 expression. The glycerophosphate (GroP) fraction prepared from chemically-degraded LTA and sn-glycerol-1-phosphate suppressed DEX-induced atrogin-1 expression, whereas the glycolipid anchor fraction of LTA did not. Heat-killed cells obtained by culturing under low-Mn2+ conditions, which generated fewer poly-GroP polymers in LTA, displayed significantly lower inhibitory activity compared to heat-killed cells grown under normal conditions. These results suggested that LTA of LAB contributed to suppressing atrogin-1 expression and that the GroP moiety of LTA was responsible for its inhibitory activity.

Key Words Lactobacillaceae, glycerophosphate, muscle atrophy, ubiquitin-proteasome system, atrogin-1/MAFbx

Skeletal muscle is the largest organ and the most important protein reservoir in the human body. Thus, skeletal muscle not only facilitates movement and support of the body but also makes a major contribution to nutrition and temperature regulation. Muscle mass, which is independent of fat mass and related cardiovascular and metabolic risk factors, is inversely associated with mortality risk and prolonged survival (1). The balance between synthesis and breakdown of myofibrillar proteins is important for maintaining muscle mass (2).

The ubiquitin-proteasome system is one of the main regulatory pathways of protein degradation in skeletal muscle, which relates to disuse muscle atrophy (3). This protein degradation pathway comprises three enzymes: ubiquitin-activating E1 enzyme, ubiquitin-conjugating E2 enzyme and ubiquitin-ligating E3 enzyme (4). Atrogin-1 is an E3 ubiquitin ligase that is specifically expressed in skeletal and cardiac muscle. The function of atrogin-1 is to regulate ubiquitin-mediated protein degradation in skeletal muscle (5). Indeed, atrogin-1 is up-regulated by synthetic glucocorticoids, such as dexamethasone (DEX) (6). Overexpression of atrogin-1 in myotubes leads to muscle loss (5, 7).

Suppression of atrogin-1 expression might be a promising strategy to attenuate muscle loss. Atrogin-1 expression is suppressed by several compounds: leucine (8), peptide PYP1 in the red seaweed Pyropia yezoensis (9), sulforaphane in cruciferous vegetables (10), conessine, a steroid alkaloid (11), resveratrol, a polyphenol (12), and ginsenoside Rg1, a steroidal glycoside found in the traditional Chinese medicine Panax ginseng (13). Previously, we showed that lactic acid bacteria (LAB) cells are a potential inhibitor of DEX-induced atrogin-1 expression (14). However, it was unclear which components of LAB contribute to the inhibitory effects on DEX-induced atrogin-1 expression.

LAB cells are surrounded by a thick cell wall consisting of peptidoglycans (PG) and teichoic acids (TAs). PG, which is a major component of the cell wall of Gram-positive bacteria, comprises β1→4-linked N-acetylmuramic acid and N-acetylglucosamine disaccharide units and the glycan chains are crosslinked by short peptides (15). TAs, which are observed in Gram-positive bacteria, can account for 50% dry weight of the cell wall (16, 17). Lactobacillus spp. usually contain two types of TAs: lipoteichoic acid (LTA), which is anchored to the cell membrane, and wall-teichoic acid (WTA), which is covalently linked to the cell wall. A typical LTA consists of poly-glycerophosphate (GroP) polymers linked to a glycolipid anchor (18, 19). The GroP-repeating units are substituted with d-alanine (d-Ala), hexose, and/or N-acetylhexosamine residues (19). A typical WTA is composed of poly-GroP or poly-(ribitol phosphate) and substituted with d-Ala and glucose residues (20). Furthermore, there are some strains that share uronic-acid-containing polysaccharide (teichuronic acids).
and neutral polysaccharides without negative charges instead of WTA (17).

Here, to elucidate the mechanism by which LAB suppresses muscle loss, we focused on cell surface components, particularly LTA. The expression of LTA on the LAB cell surface is important in examining its function. The amount of LTA in cells, including the length of the GroP chain, can be changed by environmental factors (21). The GroP backbone chain of LTA is synthesized by the successive addition of GroP residues from the membrane lipid phosphatidylglycerol as the donor catalyzed by the LTA synthase (LtaS) (22). The enzymatic activity of LtaS requires Mn$^{2+}$ (21). Thus, the amount of poly-GroP in LTA depends on the concentration of Mn$^{2+}$ in the growth medium.

LTA has been reported to have several roles in bacterial physiology including growth, cell division (22), biofilm formation (23) and stimulation of host immune cells (24). There are several lines of evidence to support the structure-function relationship of LTA. Decayed LTA, obtained by alkaline hydrolysis, failed to induce proinflammatory cytokine release (25). Furthermore, several chemically-synthesized LTA derivatives have been examined for cytokine production (26). These reports indicate that the lipid moiety of the glycolipid anchor and d-Ala substituents on the GroP polymer units are required for cytokine induction of monocytes.

The objective of this study was to elucidate the constituents of LAB that suppress DEX-induced atrogin-1 expression, focusing on cell wall components of the bacteria, particularly LTA. We examined five LAB strains, whose LTA chemical structures were reported (Table 1). In addition, we also attempted to identify which region of the LTA molecule mediates its inhibitory activity.

**MATERIALS AND METHODS**

**Bacterial strains and cultivation of bacterial cells.** Bacterial strains are listed in Table 1. All strains were provided by the Japan Collection of Microorganisms, RIKEN BRC, participating in the National BioResource Project of the MEXT, Japan. LTA structures of these strains are shown in Table 1. LAB cells were cultivated overnight in Lactobacilli MRS Broth (Becton, Dickinson and Company, Franklin Lakes, NJ). The bacterial cells were harvested by centrifugation at 200 × g at 20°C for 10 min, washed with sterilized water, killed by heating at 90°C for 30 min, and lyophilized. Low-Mn$^{2+}$ cultures were performed in Lactobacilli MRS broth without MnSO$_4$·4H$_2$O.

**Preparation of peptidoglycan.** Bacterial cells (dry weight of 6.4 g) were suspended in 80 mL of sterilized water and disrupted using a wet-type micronizing device Star Burst Mini (Sugino Machine, Toyama, Japan). After cooling to room temperature, the disrupted cell suspension was centrifuged at 4,200 × g at 4°C for 50 min. The resulting pellets were washed 3 times with sterilized water and lyophilized. The lyophilized sample was treated with 10% (v/v) trichloroacetic acid (TCA) at 100°C for 20 min, and then washed with chloroform and sterilized water twice by centrifugation at 5,900 × g at 20°C for 20 min to remove the TCA. After successive washings with ethanol and diethyl ether, the pellets were lyophilized, and used as a PG preparation.

**Purification of LTA.** LTA was purified from the supernatant of the disrupted cell suspension as described previously (24). Briefly, an equal volume of 1-butanol was added to the supernatant of the disrupted cell suspension in water. The mixture was stirred for 30 min at room temperature, and centrifuged at 5,900 × g at 20°C for 20 min. The lower aqueous layer was collected, lyophilized and dissolved in 15% (v/v) 1-propanol in 100 mM sodium acetate buffer (pH 4.7). After membrane filtration (0.45 μm pore size), the clarified material was applied to an Octyl-Sepharose 4 Fast Flow column (GE Healthcare, Little Chalfont, UK). Bound material was eluted in a stepwise manner with 15, 25, 35 and 45% (v/v) 1-propanol in 100 mM sodium acetate buffer (pH 4.7). LTA was eluted with sodium acetate buffer containing 25 and 35% (v/v) 1-propanol. Combined LTA fractions were concentrated, dialyzed against sterilized water, and lyophilized.

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**Table 1.** Strains used in this study.

| Species                  | Strain     | Culture temp (°C) | Repeating unit structure (number of units) | Substituent (substitution ratio) | Glycolipid anchor structure | References |
|--------------------------|------------|-------------------|------------------------------------------|---------------------------------|-----------------------------|------------|
| Lactobacillus gasseri    | JCM 1131T  | 37                | GroP (20–30)                             | Ala (31%)                       | (Gal-Gal-Gal-Glc)$^1$-DAG   | (33)       |
|                          |            |                   |                                          |                                 | (Gal-Gal-Gal-Glc-Acyl)$^1$-2DAG|            |
| Lactocaseibacillus rhamnosus | JCM 1136T  | 37                | GroP (40)                                | none                            | Glc-Gal-Glc-DAG             | (34, 35, 36) |
|                          |            |                   |                                          |                                 | Glc-Gal-Acyl-DAG             |            |
| Lactiplantibacillus plantarum | JCM 1149T  | 30                | GroP (110)                               | Ala (42%)                       | Hex-Hex-Hex-DAG             | (37)       |
|                          |            |                   |                                          |                                 | (Hex-Hex-Hex-Acyl)$^1$-DAG   |            |
| Lactiplantibacillus pentosus | JCM 1558T  | 30                | GroP (22)                                | Glc (10%)                       | Glc-Gal-Glc-DAG             | (34, 35)   |
|                          |            |                   |                                          |                                 | Glc-Gal-AcylGlc-DAG          |            |
| Levilactobacillus brevis | JCM 1559T  | 30                | GroP                                    | Ala, Glc, AlaGlc unknown        | unknown                     | (38)       |

AlaGlc: alanyl-glucose; DAG: diacylglycerol.

$^1$The order of Gal and Glc is unknown.

$^2$The linkage position of the hexose-bound acyl group is unknown.
Preparation of chemically-degraded LTA fractions. Selective degradation of LTA to cleave the phosphodiester bond of the GroP polymer was performed by treatment with 98% (v/v) acetic acid at 100 °C for 3 h (27). After removal of acetic acid by flash evaporation, the products were partitioned with chloroform/methanol/ water (1 : 1 : 0.9. v/v). The lower organic layer was used as the glycolipid anchor fraction, and the upper aqueous layer was used as the GroP fraction. Sodium sn-glycerol-1-phosphate was purchased from Sigma-Aldrich (St Louis, MO).

Dot blotting. A polyvinylidene fluoride (PVDF) membrane (GE Healthcare) was pre-wetted with methanol, and then soaked with phosphate-buffered saline (PBS). The samples were then spotted onto the wetted membrane. The membrane was blocked with 5% (w/v) skimmed milk in PBS containing 0.1% (v/v) polysorbate 20 for 60 min at room temperature. The membrane was incubated with a 1 : 500 dilution of mouse anti-LTA monoclonal antibody clone 55 (Hycult Biotech, Uden, The Netherlands) for 60 min at room temperature, and subsequently reacted with a 1 : 1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody (Cell Signaling Technology, Danvers, MA) for 60 min at room temperature. Specific binding was detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Effect of bacterial components on DEX-induced atrogin-1 expression in myoblasts. C2C12 mouse myoblast cells (KAC, Kyoto, Japan) were seeded into 12-well plates and cultured in Dulbecco’s minimum essential medium (DMEM) containing 4.5 g/L glucose (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (Sigma-Aldrich) plus antibiotics (100 U/mL penicillin and 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 supplemented with 2% horse serum (Invitrogen, Waltham, MA) and antibiotics for 6 d. C2C12 myotubes were treated with 1 µM DEX (FUJIFILM Wako Pure Chemical Corporation) in the presence or absence of bacterial samples for 24 h. The cells were washed with PBS and harvested. mRNA expression levels of atrogin-1 were determined by quantitative reverse transcription PCR (qRT-PCR) as described below.

qRT-PCR. Total RNA was extracted from C2C12 myotubes by using a RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany). cDNA was prepared from the RNA by using a High Capacity cDNA Reverse Transcription (random primer) Kit (Applied Biosystems, Waltham, MA). qRT-PCR was conducted using the Fast SYBR Green Master Mix (Applied Biosystems). The following oligonucleotide primers were used for amplification: 5'-ATCCCAGCACAGCAACAC-3' and 5'-GGGCAACT-GCATCCTTCC-3' for mouse atrogin-1 cDNA, 5'- ATGG-CCTTCCGTGTTCCTAC-3' and 5'-TGCGTCTTCCACCCACC- TTC-3' for mouse GAPDH cDNA (14). For each sample, GAPDH mRNA was used as a normalization control and mRNA expression levels were analyzed for fold change (ΔΔCT). All reactions were performed in triplicate.

Statistical analyses. Data analysis was performed by the Tukey test using 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

Effect of bacterial cells and their components on DEX-induced atrogin-1 expression

Initially, we examined DEX-induced atrogin-1 levels in myotubes differentiated from a murine myoblast cell line C2C12. DEX-induced expression of atrogin-1 was significantly \( (p<0.01) \) suppressed in the presence of all five heat-killed LAB strains to a similar extent (Fig. 1). Heat-killed cells \( (p<0.05) \) and LTA \( (p<0.01) \) of L. gasseri JCM 1131T suppressed atrogin-1 expression, whereas PG did not (Fig. 2). LTA derived from the five LAB strains significantly \( (p<0.01) \) suppressed DEX-induced atrogin-1 expression to a similar extent (Fig. 3). These results indicated that LTA derived from the different LAB strains shared a common inhibitory activity for atrogin-1 expression.

Effect of chemically-degraded LTA fractions on DEX-induced atrogin-1 expression

To examine which region of LTA contribute to the suppression of atrogin-1 expression, we treated C2C12 myotubes with chemically-degraded LTA fractions. The aqueous phases, which corresponded to the GroP fractions, did not significantly \( (p<0.01) \) suppress DEX-induced atrogin-1 expression, whereas the organic phases, which corresponded to the glycolipid anchor fractions, did not (Fig. 4A). Sodium sn-glycerol-1-phosphate also significantly \( (p<0.05) \) suppressed DEX-induced atrogin-1 expression (Fig. 4B).

Effect of LAB cells with reduced poly-GroP of LTA on DEX-induced atrogin-1 expression

We evaluated the effect of different amounts of poly-GroP in LTA derived from cells on the suppression of DEX-induced atrogin-1 expression. The enzymatic activity of LtaS, which generates the poly-GroP backbone chain (28), is Mn2+ dependent (21). L. gasseri JCM 1131T cells obtained by cultivating under low-Mn2+ conditions showed markedly reduced binding activity of the anti-LTA monoclonal antibody clone 55, which recognizes the poly-GroP backbone chain (29), compared with cells grown under normal culture conditions (Fig. 5A). The heat-killed cells obtained by cultivation under low-Mn2+ conditions did not show inhibitory activity for DEX-induced atrogin-1 expression, whereas those obtained by cultivation under normal conditions displayed significant \( (p<0.01) \) inhibitory activity (Fig. 5B).

DISCUSSION

The results from the present study reveal that LTA derived from LAB contributed to the suppression of DEX-induced atrogin-1 expression. Moreover, this inhi-
Fig. 1. Effects of heat-killed cells of five LAB strains (100 μg/mL) on DEX-induced atrogin-1 expression in C2C12 myotubes. Error bars show SD (n=6). Different letters represent significant differences at p<0.01 (Tukey test).

Fig. 2. Effects of heat-killed cells (HK) (100 μg/mL), LTA (100 μg/mL), and PG (100 μg/mL) of L. gasseri JCM 11317 on DEX-induced atrogin-1 expression in C2C12 myotubes. Error bars show SD (n=6). Different letters represent significant differences at p<0.05 (Tukey test).

Fig. 3. Effects of LTA (100 μg/mL) derived from five LAB strains on DEX-induced atrogin-1 expression in C2C12 myotubes. Error bars show SD (n=6). Different letters represent significant differences at p<0.01 (Tukey test).
Inhibition of Atrogin-1 by Lipoteichoic Acids

Inhibition of Atrogin-1 by Lipoteichoic Acids

Inhibition of DEX-induced atrogin-1 expression was attributable to the GroP moiety of LTA. Atrogin-1, which is an important regulator of ubiquitin-mediated protein degradation in skeletal muscle (5), is a major marker of muscle loss and disuse muscle atrophy. We previously demonstrated that heat-killed L. curvatus CP2998 cells prevented DEX-induced atrogin-1 expression in C2C12 myotubes (14). Although LTA in this study exhibited significant structural diversity (Table 1), heat-killed cells or LTA derived from the different strains attenuated DEX-induced atrogin-1 expression to a similar extent, respectively (Figs. 1 and 3). Thus, we reasoned that the common structures of LTA from the different LAB strains must play a role in the observed inhibitory activity.

LTA molecules consist of two distinct moieties, a hydrophobic glycolipid anchor and a hydrophilic GroP polymer backbone chain (18). We found that GroP fractions of chemically-degraded LTA and commercial sn-glycerol-1-phosphate suppressed DEX-induced atrogin-1 expression (Fig. 4A and B). L. gasseri JCM 1131\textsuperscript{T} cells having LTA with fewer GroP polymers obtained by culturing under low-Mn\textsuperscript{2+} conditions showed reduced inhibitory activity by comparison with those obtained under normal culture conditions (Fig. 5A and B). Under the low-Mn\textsuperscript{2+} conditions, the cell growth rate became lower, and the cell shape showed slightly longer (data not shown). Taken together, our results strongly suggest that the GroP moiety of LTA played a role in suppressing atrogin-1 expression.

Further studies are needed to elucidate the mechanism by which the GroP moiety of LTA suppresses muscle atrophy. A previous report suggested that β-glycerophosphate, the isomer of sn-glycerol-1-phosphate, induced overexpression of integrin linked kinase that provokes activation of mammalian target of rapamycin (mTOR) and autophagy reduction in C2C12 myoblasts (30). It is currently thought that mTOR is essential for the maintenance of muscle mass and function (31). mTOR activation attenuates atrogin-1 expression and efficiently counteracts the catabolic processes provoked by glucocorticoids (32). Leucine (8) and ginsenoside Rg1 (13) have been shown to inhibit muscle protein degradation via regulation of mTOR in C2C12 myotubes. The same pathway in C2C12 myotubes could be activated by the GroP moiety of LTA.
In conclusion, LAB cells display an inhibitory activity for atrogin-1 expression, which appears to be mediated through the GroP moiety of LTA. Further studies on the composition, structure and organization of the cell wall of LAB are necessary to better understand the underlying mechanism of action. These findings may be useful in the development of LAB as a novel strategy for attenuating muscle loss and disuse muscle atrophy.

**Authorship**

Research conception and design: RK, TS, SS, TH, YN and SY; experiments: RK; statistical analysis of the data: RK, TS, SS and SY; writing of manuscript: RK, TS, SS, TH, YN and SY.

**Disclosure of state of COI**

RK, SS, TH, and YN are employees of Asahi Quality and Innovations, Ltd., and research expenses and salaries were provided from this company. TS and SY received a grant from Asahi Quality and Innovations, Ltd., and research expenses and salary: SY; writing of manuscript: RK, TS, SS, TH, YN and SY.

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