

ORIGINAL

Additional effects of simultaneous treatment with C14-Cblin and celastrol on the clinorotation-induced rat L6 myotube atrophy

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Abstract: Two novel reagents, N-myristoylated Cbl-b inhibitory peptide (C14-Cblin) and celastrol, a quinone methide triterpene, are reported to be effective in preventing myotube atrophy. The combined effects of C14-Cblin and celastrol on rat L6 myotubes atrophy induced by 3D-clinorotation, a simulated microgravity model, was investigated in the present study. We first examined their effects on expression in atrogenes. Increase in MAFbx1/atrogenin-1 and MuRF-1 by 3D-clinorotation was significantly suppressed by treatment with C14-Cblin or celastrol, but there was no additive effect of simultaneous treatment. However, celastrol significantly suppressed the upregulation of Cbl-b and HSP70 by 3D-clinorotation. Whereas 3D-clinorotation decreased the protein level of IRS-1 in L6 myotubes, C14-Cblin and celastrol inhibited the degradation of IRS-1. C14-Cblin and celastrol promoted the phosphorylation of FOXO3a even in microgravity condition. Simultaneous administration of C14-Cblin and celastrol had shown little additive effect in reversing the impairment of IGF-1 signaling by 3D-clinorotation. While 3D-clinorotation-induced marked oxidative stress in L6 myotubes, celastrol suppressed 3D-clinorotation-induced ROS production. Finally, the C14-Cblin and celastrol-treated groups were inhibited decrease in L6 myotube diameter and increased the protein content of slow-twitch MyHC cultured under 3D-clinorotation. The simultaneous treatment of C14-Cblin and celastrol additively prevented 3D-clinorotation-induced myotube atrophy than single treatment. *J. Med. Invest.* 69:127-134, February, 2022

Keywords: C14-Cblin, celastrol, myotube atrophy, muscle atrophy-associated ubiquitin ligases, oxidative stress

INTRODUCTION

Skeletal muscle atrophy is one of the serious social problems in Japan, which has the highest proportion of elderly citizens of any country in the world. Furthermore, with the advancement of space development, it has become possible to stay in space for long periods of time. In the long-term unloading state, muscles atrophy and motor functions are impaired. The ubiquitin-proteasomal proteolytic system plays an important role in muscle atrophy (1). This degradation mechanism is upregulated by the impaired insulin-like growth factor-1 (IGF-1) signaling. It is necessary to improve or activate the IGF-1 signaling to overcome this issue. Among natural biomaterials, two effective reagents, Cblin/Cblin-like peptides and celastrol, were found to myotube atrophy caused by various conditions, such as simulated

microgravity conditions and glucocorticoid treatment (2, 3).

Cblin is an inhibitory peptide against casitas B-lineage lymphoma-b (Cbl-b) ubiquitin ligase (4). Cbl-b is a ubiquitin ligase for IRS-1 in atrophied skeletal muscle. Cbl-b-mediated IRS-1 degradation impaired the downstream of IGF-1 signaling, for example, dephosphorylation of forkhead box-containing protein, O subfamily 3 (FOXO3), and resulted in induction of muscle atrophy-associated ubiquitin ligases (atrogenes), such as muscle atrophy F-box protein 1 (MAFbx1)/atrogenin-1 and muscle ring finger protein-1 (MuRF-1). Cbl-b recognizes and binds to phosphorylated tyrosine⁶⁰⁸ residues of IRS-1 (3). Cblin pentapeptide DGpYMP, which mimics the phosphorylation site sequence of IRS-1, functions as decoy substrate for Cbl-b, suppressing the Cbl-b-mediated IRS-1 ubiquitination in vitro and in vivo (3). It was also found that N-myristoylated Cblin prevents muscle

Abbreviation

Akt, protein kinase B; atrogenes, atrophy-related ubiquitin ligase genes; Cbl-b, casitas B-lineage lymphoma-b; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FOXO, forkhead box O; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3, glycogen synthase kinase-3; H2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; HBSS, Hanks' balanced salt solution; HSPs, heat shock proteins; IGF-1, insulin-like growth factor-1; IRS-1, insulin receptor substrate-1; LDH, lactate dehydrogenase; MAFbx, muscle atrophy F-box protein; mTOR, mammalian target of rapamycin; MuRF-1, muscle RING finger protein-1; MyHC, myosin heavy chain; PI3K, phosphoinositide-3 kinase; qRT-PCR, quantitative reverse transcription and polymerase chain reaction; ROS, reactive oxygen species; S6K, p70 S6 kinase; 3D-clinorotation, three-dimensional-clinorotation.

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atrophy more effectively than unmodified Cblin (5). Another is celastrol, a quinone methide triterpene derived from a plant of the Euonymus family. Celastrol is a potent HSP inducer and has anti-inflammatory and antioxidant effects (4, 6). It strongly activated Akt and suppressed MuRF-1 expression in skeletal muscle. It also activated FOXO3 and promoted muscle protein synthesis (2).

Combined effects of these reagents were examined in the present study, which distinctly activated the IGF-1 signaling, on myotube atrophy to enhance the preventive effect on muscle atrophy.

MATERIALS AND METHODS

Reagents and stock solutions

C14-Cblin (GSJ, SC1208) and celastrol (Cayman, 70950) and dexamethasone (DEX) (Sigma) were dissolved in dimethyl sulfoxide (DMSO; Sigma, D8418) at stock concentrations of 100 mM.

Cell culture

Rat myoblastic L6 skeletal muscle cells were maintained and proliferated at 37°C with 5% CO₂/95% air in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixed solution (Nacalai Tesque, Kyoto, Japan). When the L6 myoblasts became 100% confluence, the medium was changed to DMEM containing 0.5% FBS, and the L6 myoblasts were fused. Cells were maintained in 0.5% FBS (differentiation medium) for 5 days. DMSO and C14-Cblin 35 μM were added to L6 myotubes and cultured in 3D-clinostat. Celastrol 500 nM was added 54 h after the start of 3D-clinorotation, and the myotubes were collected 6 h later.

3D-clinorotation

L6 myotubes were subjected to 3D-clinorotation in a 3D-clinostat (Mitsubishi Heavy Industries, Kobe, Japan) (Advanced Engineering Services, Tokyo, Japan), according to the method of Hirasaka, *et al.* (7). Briefly, 12-well plate containing L6 myotubes were filled with differentiation medium, and they were treated with 35 μM DMSO (Vehicle), 35 μM C14-Cblin, 500 nM celastrol, or both of them. They were rotated at 37°C in the 3D-clinostat apparatus in a 5% CO₂/95% air for 60 h. The rate and cycle of rotation were controlled by the computer to randomize the gravity vector both in magnitude and in direction and then

the dynamic stimulation of gravity to cells was cancelled in any direction. Sedentary cells were incubated in parallel under the condition except for the rotation.

Measurement of myotube diameters

Myotubes were photographed by phase contrast microscopy BZ-9000 (Keyence, Osaka, Japan) at 20-fold magnification. A total of 100 myotube diameters per group was measured with BZ Analyzer (Keyence). The results were expressed as a percentage of the mean diameter relative to the myotube of the control group.

Cell viability assay

Cell damage was assessed by lactate dehydrogenase (LDH) activity in the culture media. The activity was measured with the Cytotoxicity LDH Assay Kit-WST (Dojindo, Tokyo, Japan), according to the manufacturer's protocol.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was performed with the Power SYBR Green PCR Master Mix (Thermo Fischer Scientific) using an Applied Biosystems 7300 Real-time PCR system (Thermo Fischer Scientific) (8). The oligonucleotide primers used for amplification are listed in the Table.

Western blotting and immunoprecipitation

Protein concentration was determined by Lowry's method with bovine serum albumin as a standard (9). Proteins (10 μg/lane) obtained from cell homogenates were subjected to immunoblotting. Western blotting immunoprecipitation analysis was performed by the method described previously (7). The following antibodies were used: anti-IRS-1 (Calbiochem, La Jolla, CA), anti-Cbl-b (no.9498, Cell Signaling Technology), anti-p-FOXO3a (#9466, Cell Signaling Technology), anti-FOXO3a (#2497, Cell Signaling Technology), anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse IgG (M9044; Sigma), anti-rabbit IgG (no.7074, Cell Signaling Technology).

ROS detection

C14-Cblin 35 μM and celastrol 500 nM were added to L6 myotubes, and the cells were collected 2.5 days after 3D-clinorotation. As a positive control, cells were treated with 10 μM DEX at 37°C for 24 h. Cells were incubated in 0.5% FBS with 5 μM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Thermo

Table. Primer sequences for qRT-PCR

Target gene		Sequence	Length (bp)
MAFbx1/ atrogin-1 (rat)	S	5'-AGATCCGCAAGCGATTGATC-3'	84
	AS	5'-TTGGGTAACATCGCACAAAGC-3'	
MuRF-1 (rat)	S	5'-TGTCTGGAGGTCGTTTCCG-3'	182
	AS	5'-CTCGTCTTCGTGTTCTTGC-3'	
GAPDH (rat)	S	5'-CGTGTTCCCTACCCCAATGT-3'	74
	AS	5'-ATGTCATCATACTTGGCAGGTTTCT-3'	
HSP70 (rat)	S	5'-CAGGATTTGCCCTATCCAGA-3'	150
	AS	5'-CTCTACTAAGGCCGCACTGG-3'	
Cbl-b (rat)	S	5'-GAAGGGTGAAGATGCTTTTGATG-3'	333
	AS	5'-TGATGAATTTCTGGTGCAGTCCT-3'	

AS, antisense primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; S, sense primer.

Fisher Scientific). After incubation for 1 h, cells were washed by HBSS twice and fluorescence was measured by Infinite M Nano+ plate reader (TECAN).

Capillary-based electrophoresis

Proteins were measured using a capillary-based electrophoresis instrument, WES system (ProteinSimple, San Jose, CA), according to the manufacturer's instructions. 12–230 kDa Separation Module (ProteinSimple SM-W004) and Anti-Mouse Detection Module (ProteinSimple DM-002) were used for protein detection. The data were analyzed with the Compass software for Simple Western (ProteinSimple, San Jose, CA).

Statistical analysis

All data were expressed as the mean ± SD of three to five individual samples per group. Differences between groups were analyzed via one-way analysis of variance with a software Excel Toukei Ver.7.0 (Esumi Co. Ltd. Tokyo, Japan). Differences

between two groups were tested with Scheffé's test. $P < 0.05$ was considered statistically significant.

RESULTS

Changes in the diameter and atrogenes expression of L6 myotubes by the 3D-clinorotation

L6 myotubes treated with C14-Cblin and celastrol or both were subjected to 3D-clinorotation for 3 days; the diameter and atrogenes, such as MAFbx/atrogen-1, MuRF1, and Cbl-b, of the myotubes were measured. The 3D-clinorotation significantly decreased the diameter of L6 myotubes by 10%, compared with the control (the diameter of L6 myotubes before 3D-clinorotation) (Fig. 1A). The expression in atrogenes was also upregulated by the 3D-clinorotation significantly. Their upregulated expression showed the peak value on day 2.5, day 3, and day 2.5, respectively, after the 3D-clinorotation (Fig. 1B). The tested parameters

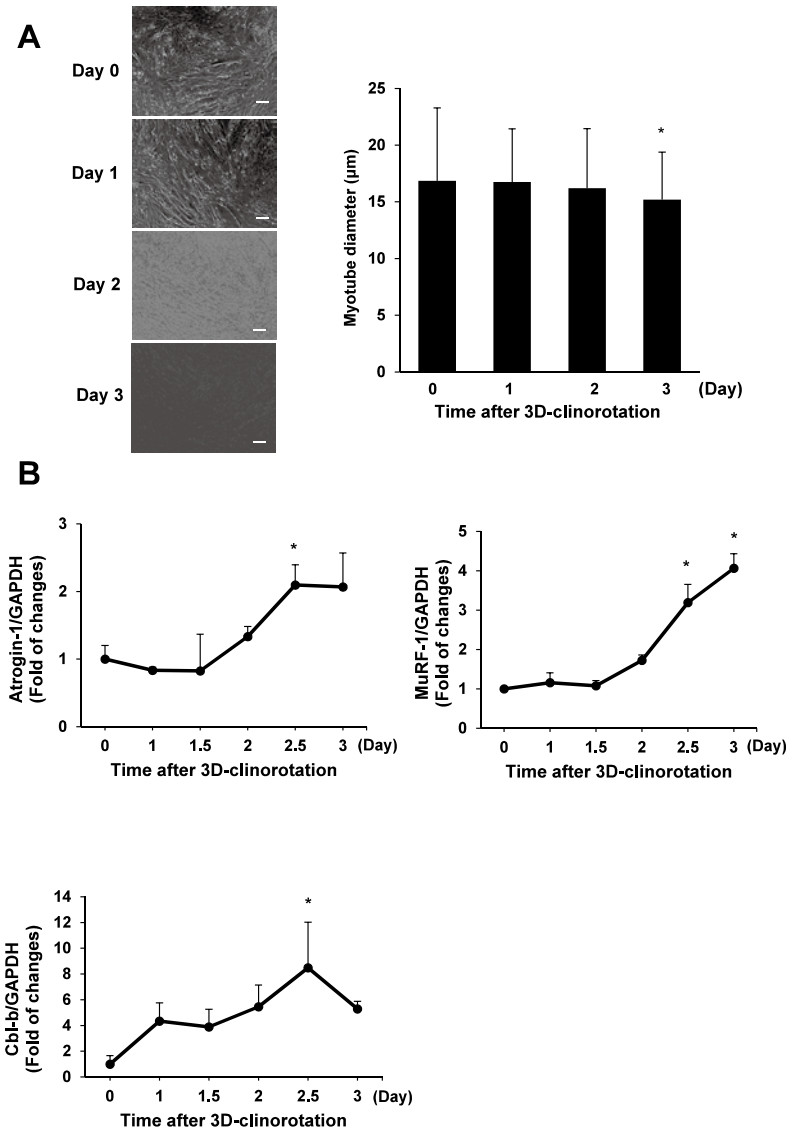


Fig 1. Myotube atrophy and amounts of mRNA levels of L6 cells cultured with the 3D-clinorotation. After 3D-clinorotation for 3 days, L6 myotube diameter decreased and increased expression of the muscle atrophy-related genes MAFbx1/atrogen-1 and MuRF-1, ubiquitin ligase Cbl-b. (A) Myotube diameters were measured daily for 3 days. The left shows myotubes of a randomly selected field. Scale bar = 100 µm. Results are mean ± standard deviation; n = 100 per group. * $P < 0.05$ compared to day 0. (B) After the cells were subjected to 3D-clinorotation for 3 days, gene expression was observed. Each line graph shows the expression variation of each gene. The vertical axis shows the expression ratio of each gene after 3D-clinorotation. Data are mean ± standard deviation (n = 3). * $P < 0.05$ compared to day 0.

were examined in L6 myotubes on 2.5 to 3 days after 3D-clinorotation based on the above results.

Inhibitory effects of C14-Cblin and / or celestrol on the 3D-clinorotation-induced expression of atrogenes in L6 myotubes

Free cell toxicity of the simultaneous treatment of C14-Cblin and celestrol was confirmed first. The optimal concentrations and period (35 μ M for 2.5 days and 500 nM for 6 h, respectively) of single treatment with C14-Cblin or celestrol against myotube atrophy (data not shown) were used. No significant increase in LDH was observed in respective single treatment and simultaneous treatment (Fig. 2A), indicating that the simultaneous treatment at the indicated concentration was not toxic.

Next, whether the simultaneous treatment with C14-Cblin and celestrol additively suppressed the 3D-clinorotation-mediated expression of the muscle atrophy-related genes (Fig. 2B) was investigated. No additive or synergistic effect of the simultaneous

treatment was observed, whereas the 3D-clinorotation-induced expression of MAFbx1/atrogin-1 and MuRF-1 was significantly suppressed by single treatment with C14-Cblin or celestrol. These findings led to the conclusion that only single treatment was enough to inhibit the 3D-clinorotation-upregulated expression of these atrogenes. Since Cblin is inhibitory peptide against Cbl-b-mediated ubiquitin (3), it was rational that C14-Cblin did not affect the expression of Cbl-b in L6 myotubes. Interestingly, celestrol significantly inhibited the 3D-clinorotation-induced expression of Cbl-b, and the simultaneous treatment hardly enhanced its suppression. Since celestrol is a potent HSP70 inducer (4), the HSP70 expression was examined further to elucidate the effect of the simultaneous treatment. Celestrol treatment of L6 myotubes significantly upregulated HSP70 expression, whereas its simultaneous treatment showed no additional effect on HSP70 expression.

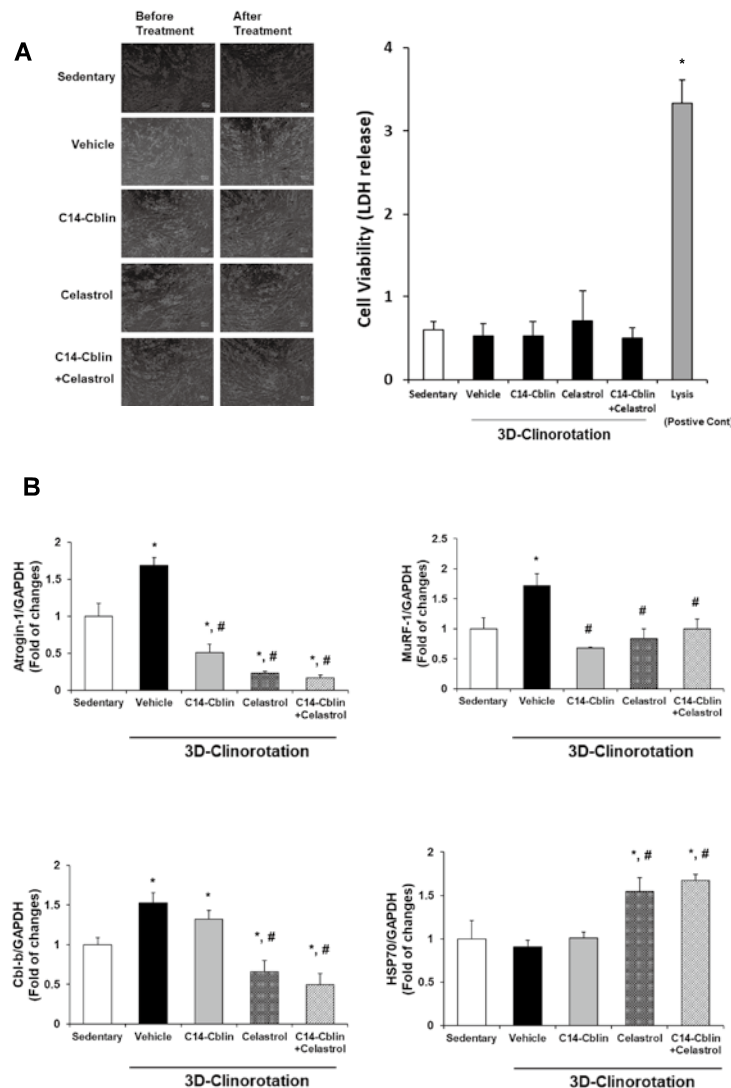


Fig 2. Inhibitory effects of C14-Cblin and celestrol on 3D-clinorotation-induced atrogenes expression in L6 myotubes. L6 myotubes were treated with 35 μ M C14-Cblin at the start of 3D-clinorotation and 500 nM celestrol 6 h before collecting. Cells were collected 2.5 days after 3D-clinorotation. (A) Assessment of myotube viability by C14-Cblin and celestrol. Collected myotubes were subjected to LDH assay; the effect of each reagent on L6 myotube viability was examined. Left, each reagent was treated and before and after that compared and observed. Scale bar = 100 μ m. Right, cell viability by LDH cytotoxicity assay. The cells treated with lysis buffer are used as positive controls. Data are mean \pm standard deviation (n = 8). * $P < 0.05$ compared to sedentary. (B) C14-Cblin and celestrol prevent 3D-clinorotation-induced expression of atrogenes in L6 myotubes. Levels of atrogenes (MAFbx1/atrogin-1, MuRF-1) and HSP70, Cbl-b transcripts were assessed by real-time RT-PCR. The ratios of target transcript levels to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were calculated. Data are mean \pm standard deviation (n = 3~4). * $P < 0.05$ compared to sedentary; # $P < 0.05$ compared to vehicle.

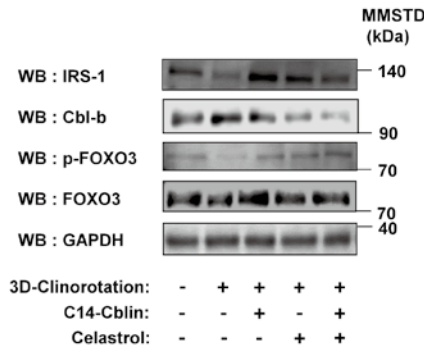
Effects of C14-Cblin and/or celastrol on IGF-1 signaling impaired by 3D-clinorotation in L6 myotubes

Since the 3D-clinorotation-induced Cbl-b-mediated IRS-1 degradation and impaired the IGF-1 signaling in myotubes (3, 10), we examined effects of C14-Cblin and/or celastrol on IGF-1 signaling (Fig. 3A). Although the 3D-clinorotation decreased the level of IRS-1 in L6 myotubes, C14-Cblin effectively prevented its degradation. Celastrol prevented the degradation of IRS-1 because Cbl-b expression was suppressed by its treatment, which is consistent with the results of quantitative RT-PCR (Fig. 2B). The simultaneous treatment with C14-Cblin and celastrol hardly showed additional effects to restore the 3D-clinorotation-mediated impairment of IGF-1 signaling. Although C14-Cblin and celastrol reactivated FOXO3a inhibited (dephosphorylated) by 3D-clinorotation, there was no additional effect on FOXO3a phosphorylation by the simultaneous treatment.

Effects of C14-Cblin and/or celastrol on 3D-clinorotation-mediated oxidative stress

It has been reported that Cbl-b expression was upregulated by oxidative stress (11). Unexpectedly, celastrol effectively prevented the 3D-clinorotation-mediated Cbl-b expression (Figs. 2B and 3A). To elucidate its mechanism, the effect of celastrol on oxidative stress in L6 myotubes was measured (Fig. 3B). The 3D-clinorotation-induced significant oxidative stress in L6 myotubes. Interestingly, it was observed that celastrol suppressed the 3D-clinorotation-induced ROS production in L6 cells whereas C14-Cblin did not affect it. These findings suggested that celastrol downregulated Cbl-b expression through its anti-oxidative effect.

A



B

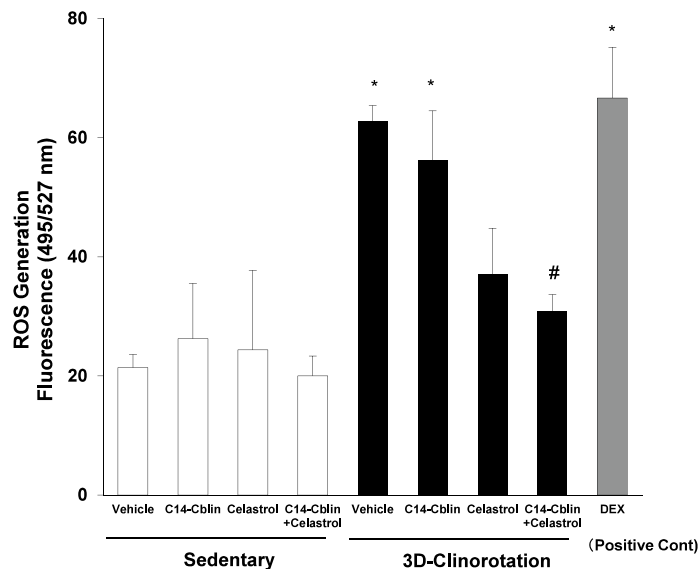


Fig 3. Effects of C14-Cblin and celastrol on IGF-1 signaling impaired by 3D-clinorotation in L6 myotubes (A) C14-Cblin and celastrol restored the inhibition of IGF-1 signaling by 3D-clinorotation. L6 myotubes were treated with 35 μM C14-Cblin, 500 μM celastrol, or both. They were subjected to 3D-clinorotation for 2.5 days. Whole cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blotting (WB) to visualize the indicated proteins. (B) Celastrol suppressed the increase in ROS levels. Each reagent-treated L6 myotubes were subjected to 3D-clinorotation for 2.5 days and then cells were collected. Cells were incubated in 0.5% FBS with 5 μM H2DCFDA. After incubation for 1 h, fluorescence was measured. Cells treated with 10 μMDEX at 37°C for 24 h were used as positive controls. **P* < 0.05 compared to vehicle of sedentary ; #*P* < 0.05 compared to vehicle subjected to 3D-clinorotation.

Effects of C14-Cblin and/or celastrol on inhibition effect of C14-Cblin and celastrol on 3D-clinorotation-induced L6 myotube atrophy

It was examined whether C14-Cblin and celastrol further inhibit 3D-clinorotation-induced myotube atrophy. It was found that L6 myotubes subjected to 3D-clinorotation for 3 days were decreased the thickness by 13%, whereas myotubes treated with C14-Cblin and celastrol were decreased by only 4% (Fig. 4A). In addition, the simultaneous treatment of C14-Cblin and celastrol reduced only by 2%.

Unloading conditions preferentially decreased slow-type MyHC levels in skeletal muscle resulting in its atrophy (12). Therefore, slow-type MyHC in L6 myotubes cultured under the 3D-clinorotation were finally measured (Fig. 4B). The 3D-clinorotation for 2.5 days decreased the amount of slow-type MyHC. Single treatment with C14-Cblin or celastrol restored it, while the efficacy of celastrol was much stronger than that of C14-Cblin. The simultaneous treatment with C14-Cblin and celastrol further increased the level of slow-type MyHC.

These results suggest that C14-Cblin and celastrol suppressed muscle atrophy by 3D-clinorotation, and the simultaneous

treatment with C14-Cblin and celastrol additively prevented the 3D-clinorotation-mediated myotube atrophy.

DISCUSSION

It has been reported that oral intake of rice overexpressing ubiquitin ligase inhibitory pentapeptide, C14-Cblin, prevents atrophy in denervated skeletal muscle (13) and inhibition of C2C12 myotube atrophy by a novel HSP70 inducer, celastrol, via activation of Akt1 and ERK1/2 pathways (2), as shown in Fig. 5. This study investigated the additive effectiveness of these antimyotube atrophy compounds on myotube atrophy caused by the 3D-clinorotation, which is mimic to the space environment. The present results showed that as far as estimated by the myotube diameter and the amount of slow-type MyHC, the antiatrophic effect was additively enhanced by their simultaneous treatment.

C14-Cblin and celastrol stimulated the IGF-1 signaling by inhibiting Cbl-b-mediated IRS-1 degradation and inducing HSP70 proteins, respectively (Fig. 5). However, the present results failed to show the additive effects of the simultaneous treatment of the IGF-1 signaling, such as phosphorylation of FOXO and

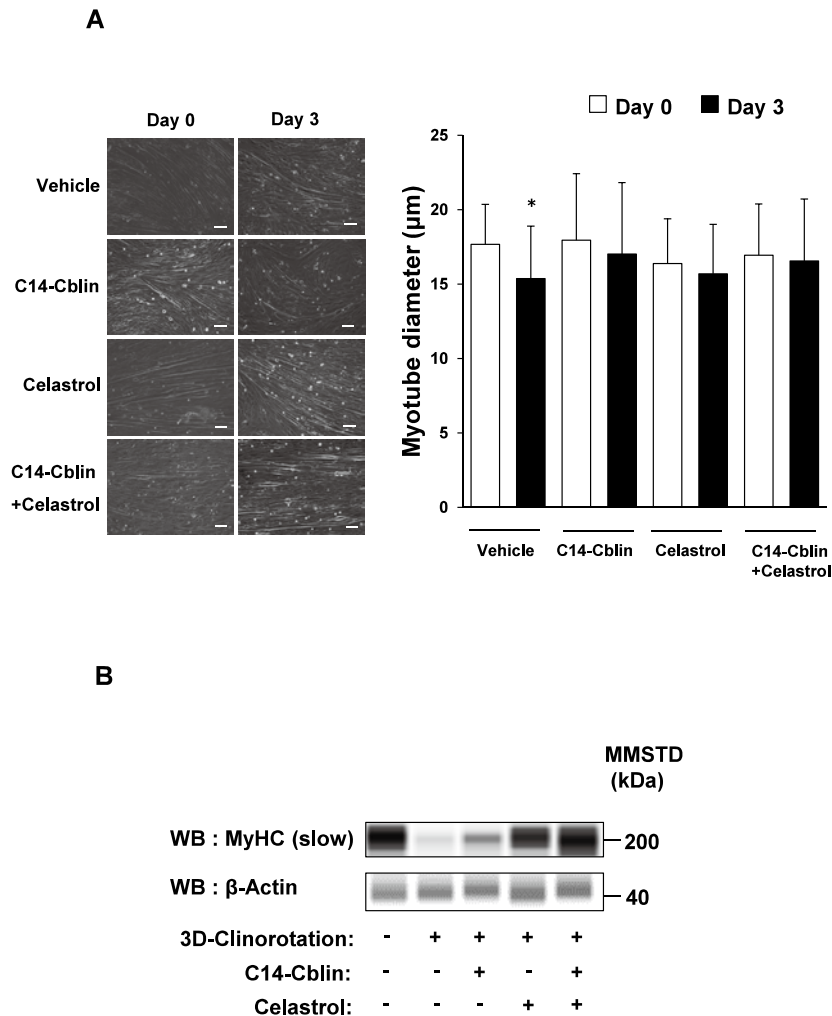


Fig 4. C14-Cblin and celastrol have a synergistic effect on the inhibition of 3D-clinorotation-induced L6 myotubes atrophy (A) L6 myotubes were treated with 35 µM C14-Cblin, 500 µM celastrol, or combined. After 3D-clinorotation for 3 days, cells were collected and observed. The left shows myotubes of a randomly selected field. Scale bar = 100 µm. Right, measurement of diameters. The result is the mean ± standard deviation. n = 50 per group. * $P < 0.05$ compared to vehicle day 0. (B) L6 myotubes were treated with C14-Cblin or celastrol or combined. L6 myotubes were subjected to 3D-clinorotation for 2.5 days. Whole cells were subjected to capillary electrophoresis with the instrument of the WES system.

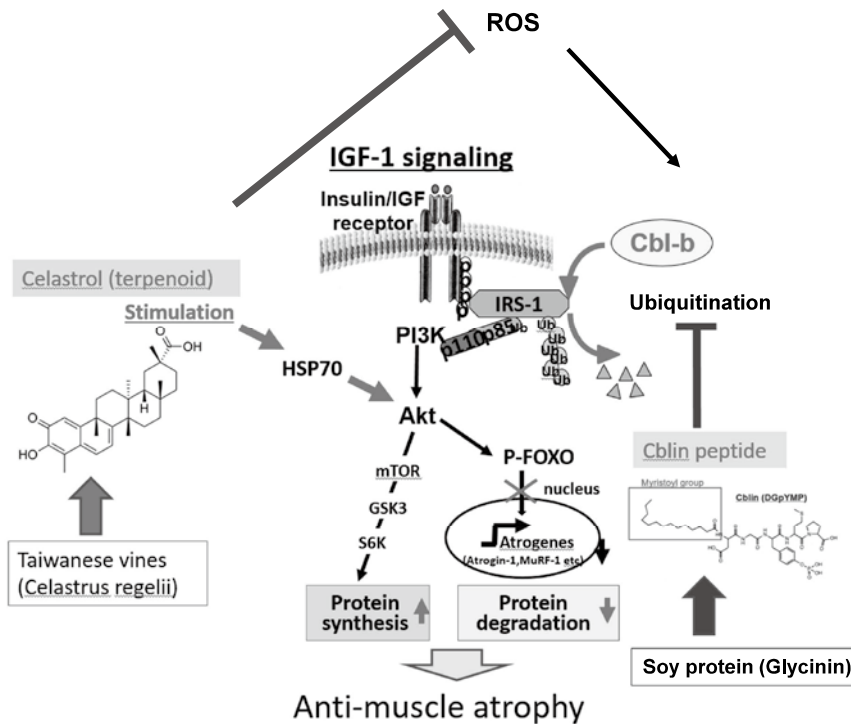


Fig 5. A model for signal induction by C14-Cblin and celastrol in the IGF-1 signaling pathway. Insulin-like growth factor 1 (IGF-1) signaling is a major pathway of skeletal muscle growth and hypertrophy. We found that C14-Cblin inhibits the binding of Cbl-b to IRS-1 and suppresses muscle atrophy under unloading condition in L6 myotubes. WeIt was also reported that celastrol activates protein anabolic signaling by upregulating the expression of HSP70 and suppressed 3D-clinorotation-induced Cbl-b upregulation by inhibiting ROS. Finally, it was found that simultaneous administration of these reagents under microgravity conditions inhibited muscle atrophy in an additive manner. Cblin/Cblin-like peptides are similar to the specific portion of soy protein glycinin (3, 15). C14-Cblin is a myristoylated Cblin peptide, which has more highly inhibitory activity against ubiquitin ligase Cbl-b than Cblin (3). Celastrol is extracted from the root of the *Celastraceae* (2, 16). These novel reagents are naturally occurring inhibitors of muscle atrophy, suggesting the possibility of combining various natural substances.

expression of atrogenes. Because L6 cells were treated with C14-Cblin and celastrol at the optimal concentration for the most adequate period, even single treatment showed the maximal effect on these parameters of the IGF-1 signaling. Validity period for stimulating the IGF-1 signaling may be longer by the simultaneous treatment.

Western blotting of slow-type MyHC showed the efficacy of celastrol was much stronger than that of C14-Cblin. It was found that celastrol effectively suppressed the 3D-clinorotation-induced Cbl-b expression through its antioxidative effects. It was also observed that celastrol functioned as a proteasome inhibitor, besides a HSP70 inducer (14). These functions possibly make celastrol a stronger effector on unloading-mediated myotube atrophy.

In an aging society, the bedridden population is expected to increase. Cblin-like peptides are derived from a soy protein, glycinin (15), and celastrol is extracted from the root of the *Celastraceae* (16). Combining such natural products and ingesting them as meals and medicines will help maintain and improve the health of the Japanese people in an aging society as well as astronauts living in space for a long time.

CONFLICT OF INTEREST

There is no conflict of interest.

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GRANT

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