ORIGINAL

Polyphenols prevent clinorotation-induced expression of atrogenes in mouse C2C12 skeletal myotubes

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Abstract : Oxidative stress is a key factor in stimulating the expression of atrogenes, which are muscle atrophy-related ubiquitin ligases, in skeletal muscle, and it induces muscle atrophy during unloading. However, the effects of antioxidative nutrients on atrogene expression have not been demonstrated. We report on the inhibitory effects of polyphenols, such as epicatechin (EC), epicatechin gallate (ECg) and epigallocatechin gallate (EGCg) and quercetin, on atrogene expression up-regulated by three dimensional (3D)-clinorotation or glucocorticoid. These treatments markedly elevated the expression of atrogenes, including atrogin-1 and MuRF-1, in mouse C2C12 myoblasts and myotubes. Interestingly, EC, ECg, EGCg and quercetin significantly decreased the expression of atrogin-1 and MuRF-1 up-regulated by 3D-clinorotation, whereas they hardly affected atrogene expression induced by dexamethasone. ERK signaling is a well known MAPK pathway to mediate oxidative stress. Therefore, we also investigated the effect of these polyphenols on phosphorylation of ERK in C2C12 myotubes. As expected, EC, ECg, EGCg, and quercetin significantly suppressed phosphorylation of ERK, corresponding to the up-regulation of atrogenes induced by 3D-clinorotation. These results suggest that antioxidative nutrients, such as catechins and quercetin, suppress atrogene expression in skeletal muscle cells, possibly through the inhibition of ERK signaling. Thus, catechins and quercetin may prevent unloading-mediated muscle atrophy. J. Med. Invest. 56 : 26-32, February, 2009

Keywords **:** *atrogenes, dexamethasone, mouse C2C12 cells, polyphenols, 3D-clinorotation*

INTRODUCTION

Unloading, as occurs in long-term bed rest, immobilization, and weightlessness, causes rapid and marked muscle atrophy (1, 2). We previously reported that unloading, including spaceflight as well as tail-suspension, stimulated ubiquitination of various proteins, including myosin heavy chain (MHC), and accumulation of MHC degradation fragments in atrophied rat gastrocnemius muscle (3). Recent studies demonstrated that two ubiquitin-protein ligases (E3s), atrogin-1/MAFbx and MuRF1, are critical in

Abbreviations : ANOVA, analysis of variance ; DMEM, Dulbecco's modified Eagle's Medium ; E2, ubiquitin conjugate enzyme ; E3, ubiquitin ligase ; EC, epicatechin ; ECg, epicatechin gallate ; EGCg, epigallocatechin gallate ; ERK, extracellular-signal regulated kinase ; MHC, myosin heavy chain ; PCR, polymerase chain reaction ; ROS, reactive oxygen species ; RT, reverse transcription ; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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the development of muscle atrophy (4, 5). Atrogin-1 and MuRF-1 are induced early on in the atrophy process, and the rise in atrogin-1 and MuRF-1 expression precedes the loss of muscle weight (6-8). Indeed, animals lacking atrogin-1 and MuRF-1 genes are resistant to muscle atrophy caused by denervation (4). Based on these findings, we hypothesized that inhibition of atrogin-1 and MuRF-1 expression may prevent or reduce muscle atrophy.

Muscle atrophy, caused by immobilization or unloading, is associated with oxidative stress and the generation of reactive oxygen species (ROS) (9). In our previous study, exogeneous ROS significantly upregulated the expression of muscle atrophy related ubiquitin ligase (10). Thus, oxidative stress may serve as an important trigger of signaling pathways leading to muscle atrophy during prolonged periods of disuse. In fact, supplementation of cysteine, an antioxidative nutrient, prevented unweighting-induced ubiquitination in association with redox regulation in rat skeletal muscle (11). However, other antioxidative nutrients have not been reported to prevent activation of muscle atrophy related-ubiquitin ligase.

This study was designed to examine the antioxidative effects of polyphenols, such as tea catechins and quercetin, on oxidative stress-mediated or unrelated expression of atrogenes, in mouse C2C12 myoblasts and myotubes. We examined the inhibitory effect of polyphenols on the expression of atrogenes subjected to three-dimensional (3D)-clinorotation or treated with dexamethasone. Catechins and quercetin decreased the expression of atrogenes induced by oxidative stress. Our results suggest that supplementation of antioxidative nutrients, such as catechins and quercetin, might be beneficial in the prevention of muscle atrophy caused by oxidative stress.

MATERIALS AND METHODS

Cell culture

C2C12 myoblastic cells were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). C2 C12 myoblastic cells were maintained and proliferated at 37°C with 5% CO_2/95% air in Dulbecco's modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. At a confluence of 100%, C2C12 myoblastic cells were fused by shifting medium to DMEM containing 2% horse serum. Cells were maintained in 2% horse serum (differentiation medium) for 96 h. At 72 h, cells were treated with 100 μM dexamethasone and 125 μM antioxidative nutrients such as, epicatechin (EC), epicatechin gallate (ECg) and epigallocatechin gallate (EGCg) and quercetin for 24 h.

3D-clinorotation

We subjected C2C12 myotubes to 3D-clinorotation in a 3D-clinostat (Mitsubishi Heavy Industries, Kobe, Japan), according to the method of Hirasaka, *et al.* (12). Briefly, flasks containing C2C12 myotubes were filled with DMEM containing 2% horse serum. Three days later, they were rotated at 37°C in the 3D-clinostat apparatus in a 5%-CO_2 chamber. 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. Control cells were incubated in parallel under the same conditions except for the rotation.

Real-time reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from whole cells according to standard protocols (13). Real-time RT-PCR with SYBR™ Green dye was performed using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA), as described previously (14). The following oligonucleotide primers were used for amplification : 5'-GGCGGACGGCTGGAA-3' and 5'-GGCGGACGGCTGGAA-3' for mouse atrogin-1 cDNA ; 5'-ACGAGAAGAAGAGCGAGCTG-3' and 5'-CTTGGCACTTGAGAGAGGAAGG-3' for mouse MuRF-1 cDNA ; 5'-ACCCAGAAGACTGTGGATG-G-3' and 5'-TTCAGCTCTGGGATGACCTT-3' for mouse GAPDH cDNA.

Immunoblot analysis

Immunoblot analysis was performed as described previously (14). Whole-cell extracts (40 μg protein/lane) were subjected to SDS-10%-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 4% skimmed milk and then incubated with primary antibodies for 1 h at 25°C. The following primary antibodies were used : anti-phospho-ERK and anti-ERK (Cell Signaling Technology, Danvers, MA). The bound antibodies were detected with suitable secondary antibodies and the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK). Protein concentrations were

measured using Lowry's method (15).

Statistical analysis

All data were statistically evaluated by analysis of variance (ANOVA) with SPSS software (release 6.1 ; SPSS Japan Inc., Tokyo, Japan) and were expressed as mean± SD, n=3. Individual differences between groups were assessed with Duncan's multiple range test. Differences were considered significant at $P<0.05$.

RESULTS

Effects of catechins and quercetin on 3D-clinorotation-induced atrogene expression in C2C12 myoblasts and myotubes

In our previous study, we demonstrated that unloading, such as spaceflight, tail-suspension and denervation, significantly upregulated ubiquitin ligase expression in skeletal muscle (10). We, therefore, examined whether antioxidative polyphenols suppress the expression of atrogenes such as atrogin-1 and MuRF-1, in C2C12 myoblasts or myotubes subjected to 3D-clinorotation. In both C2C12 myoblasts and myotubes, the expression of atrogin-1 and MuRF-1 was significantly induced by 6 h of 3D-clinorotation (Fig. 1). Upregulation of atrogin-1 expression in response to 3D-clinorotation was greater than the upregulation of MuRF-1 expression. Interestingly, treatments of all polyphenols used in this study significantly decreased the amounts of atrogin-1 and MuRF-1 transcripts caused by 3D-clinorotation (Fig. 1). Treatment with all tested polyphenols did not affect the expression of atrogenes in C2C12 myoblasts and myotubes without 3D-clinorotation (data not shown).

Effects of polyphenols on dexamethasone-induced atrogene expression in C2C12 myoblasts and myotubes

We also examined the effects of polyphenols on dexamethasone-induced expression of atrogenes in C2C12 myoblasts or myotubes. Dexamethasone is a well-established chemical reagent known to induce expression of atrogenes without oxidative stress (16). Dexamethasone up-regulated the expression of atrogin-1 and MuRF-1 transcripts (Fig. 2). In this

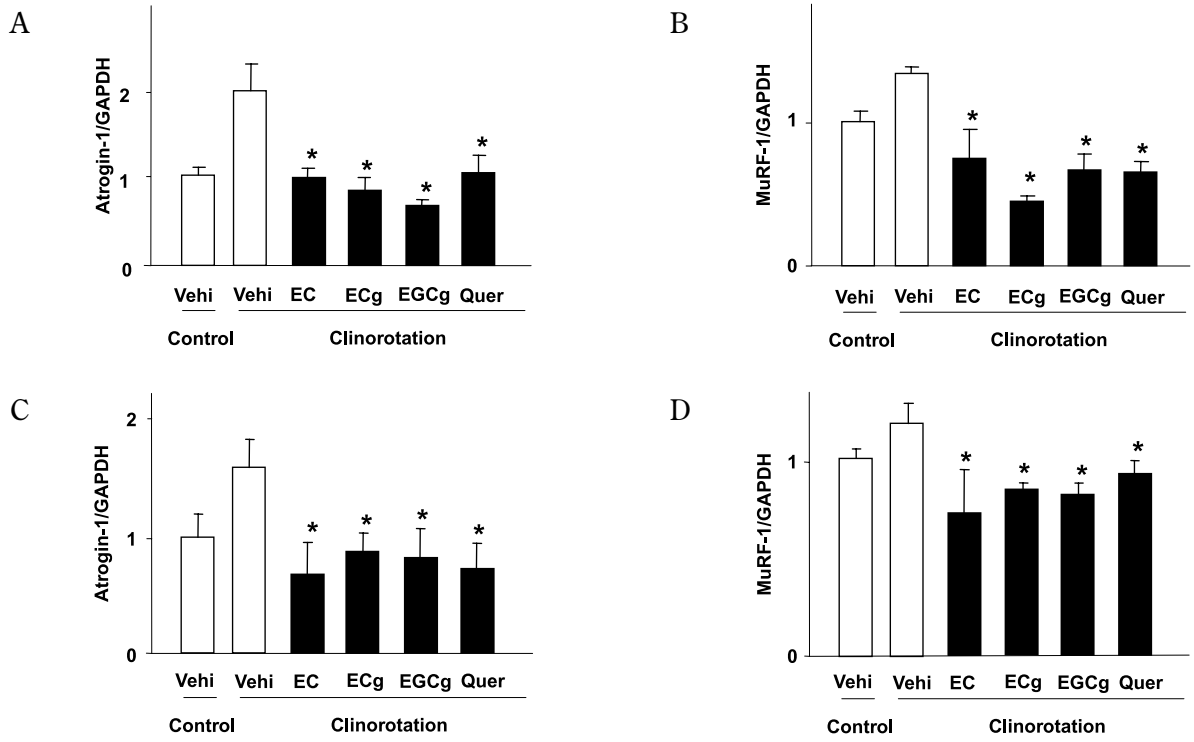

Fig. 1. Effect of polyphenols on C2C12 cells, subjected to three-dimensional (3D)-clinorotation, cultured with a proliferating (A, B) or differentiating medium (C, D). C2C12 cells were cultured as described in MATERIALS AND METHODS. Cells were cultured in proliferation or differentiation media for 72 h, before being subjected to 3D-clinorotation. Cells were pretreated with polyphenols (125 μM), or quercetin (125 μM), for 1 h. Total RNA was extracted from C2C12 cells and real-time RT-PCR for atrogin-1, MuRF-1 and GAPDH was performed as described in MATERIALS AND METHODS. The intensity ratios of cDNA of atrogin-1 and MuRF-1 to GAPDH were calculated. Each mRNA level was standardized by that of GAPDH. The values are means± S.D. (n=3). *$P<0.05$, compared with the value of rotated cells. EC, epicatechin ; ECg, epicatechin gallate ; EGCg, epigallocatechin gallate ; quer, quercetin ; Vehi, Vehicle.

experiment, EC, ECg, EGCg, and quercetin failed to inhibit dexamethasone-induced upregulation of atrogin-1 and MuRF-1 expression in C2C12 myoblasts and myotubes (Fig. 2).

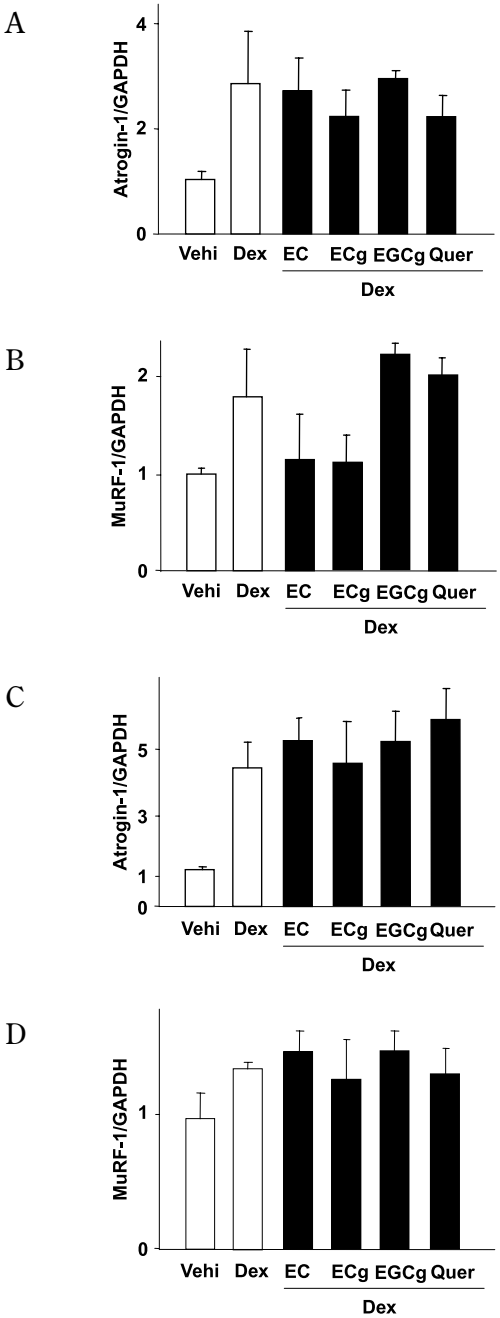

Fig. 2. Effect of polyphenols in C2C12 cells treated with dexamethasone cultured with a proliferating (A, B) or differentiating medium (C, D). After culturing with proliferation or differentiation media for 72 h, cells were treated with 100 μM dexamethasone (Dex) and polyphenols, such as 125 μM catechins or 125 μM quercetin, for 24 h. Total RNA was extracted from C2 C12 cells. Real-time RT-PCR for atrogin-1, MuRF-1 and GAPDH was performed as described in MATERIALS AND METHODS. The intensity ratios of cDNA of atrogin-1 and MuRF-1 to GAPDH were calculated. Each mRNA level was standardized by that of GAPDH. The values are means± S.D. (n=3). *P< 0.05, compared with the value of dexamethasone-treated cells. Dex, dexamethasone. EC, epicatechin ; ECg, epicatechin gallate ; EGCg, epigallocatechin gallate ; quer, quercetin ; Vehi, Vehicle.

Effects of polyphenols on 3D-clinorotation-induced phosphorylation of ERK in C2C12 myotubes

It has been reported that catechins and quercetin inhibited phosphorylation of ERK in the MAPK signaling pathway in 3T3-L1 adipocytes, rat glioma C6 cells and mesangial cells (17-19). Clinorotation induced phosphorylation of ERK in C2C12 myotubes. Phosphorylation reached its peak value 30 minutes after clinorotation (Fig. 3) and gradually returned to the basal value within 2 h (data not shown). Pretreatment with EC, ECg, EGCg and quercetin significantly inhibited the 3D-clinorotation-induced phosphorylation of ERK in C2C12 myotubes (Fig. 3).

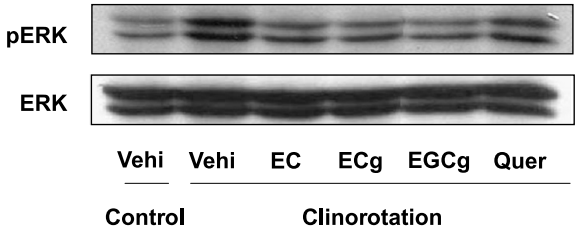

Fig. 3. Effect of polyphenols on phosphorylation of ERK in C 2C12 myotubes. After culturing with differentiation media for 72 h, cells were subjected to 3D-clinorotation for 30 min. Vehicle (Vehi) or polyphenols (125 μM) were added to all cells 1h prior to 3D-clinorotation. Protein (40 μg/lane) extracted from C2C12 myotubes were subjected to immunoblotting for phosphorylated- ERK and ERK, as described in MATERIALS AND METHODS. Similar results were obtained in three separate experiments. EC, epicatechin ; ECg, epicatechin gallate ; EGCg, epigallocatechin gallate ; quer, quercetin.

DISCUSSION

We investigated potential inhibitory effects of nutrients on skeletal muscle atrophy by focusing on the potent antioxidant properties of polyphenols, since oxidative stress is involved in the expression of muscle atrophy-related ubiquitin ligases (atrogenes) (20). Polyphenols, such as catechin and quercetin, are potent scavengers of hydroxyl radicals (OH·). Catechins, a primary constituent of green tea, reduce the risk of a variety of oxidative stress-related diseases, including prostate, breast, chronic gastritis and stomach cancer (21-23). Quercetin, a major flavonoid in onion, is a more effective antioxidant nutrient than vitamin C, vitamin E and β-carotene (24). As expected, our studies showed that catechins and quercetin effectively prevented unloading-mediated expression of atrogenes. Thus,

polyphenols are potentially effective nutrients in the prevention of muscle atrophy. In fact, our earlier studies demonstrated that intraperitoneal injection of a high dose of EGCg prevented skeletal muscle atrophy in mdx mice, a mild phenotype model of human Duchenne-type muscular dystrophy (25).

Polyphenols failed to prevent an increase in atrogin-1 and MuRF-1 transcripts expression in C2C12 myoblasts and myotubes treated with dexamethasone. Dexamethasone and 3D-clinorotation induce muscle atrophy by up-regulation of transcription of atrogenes. Since the atrogin-1 promoter lacks glucocorticoid-response elements (26), glucocorticoid has been reported to stimulate transcription of atrogenes through the activation of Foxo1 and Foxo3, the main transcription factors for atrogenes in muscle (27). These findings suggest that oxidative stress, or ROS, is not involved in glucocorticoid-mediated expression of atrogenes. In contrast, Qu, *et al* reported that 3D-clinorotation induced accumulation of oxidative stress in PC12 neural cells (28), indicating that 3D-clinorotation regulates the expression of atrogenes in a distinct mechanism to glucocorticoid. Thus, we conclude that polyphenols are not effective in preventing dexamethasone-induced expression of atrogenes.

Oxidative stress activates the ERK signaling pathway in a variety of cell types, including endothelial, fibroblastic, cardiac and smooth muscle cells (29-32). To elucidate the mechanism of beneficial effects of polyphenols on muscle atrophy, we examined their effects on ERK signaling. In this study, we found that EC, ECg, EGCg and quercetin significantly suppressed 3D-clinorotation-induced phosphorylation of ERK in C2C12 myotubes. More recently, we found that expression of another atrogene, Cbl-b, is regulated by ERK-signaling (unpublished data). These findings suggest that polyphenols may down-regulate the expression of atrogenes through inhibition of the ERK signaling. Further investigation is necessary to elucidate this hypothesis.

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