# Quercetin Prevents Unloading-Derived Disused Muscle Atrophy by Attenuating the Induction of Ubiquitin Ligases in Tail-Suspension Mice

Rie Mukai,[†] Reiko Nakao,[‡] Hironori Yamamoto,[§] Takeshi Nikawa,[‡] Eiji Takeda,[§] and Junji Terao*[,†]

*Department of Food Science, Department of Nutritional Physiology, and Department of Clinical Nutrition, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima 770-8503, Japan*

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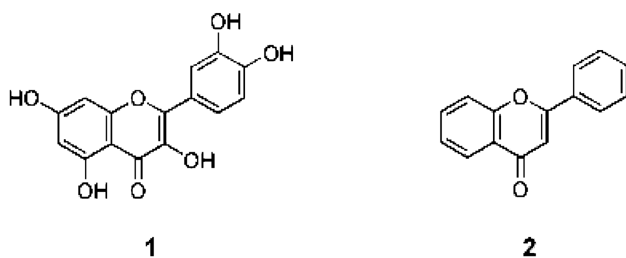
The effects of quercetin (**1**) were investigated on disused muscle atrophy using mice that underwent tail suspension. Periodic injection of **1** into the gastrocnemius muscle suppressed muscle weight loss and ubiquitin ligase expression. Compound **1** reduced the enhancement of lipid peroxidation in the muscle. Injection of *N*-acetyl-L-cysteine, but not flavone (**2**), also prevented muscle weight loss and enhancement of lipid peroxidation. These findings demonstrate that **1** can prevent disused muscle atrophy by attenuating the expression of ubiquitin ligases and that such prevention originates from its antioxidant activity.

3,5,7,3′,4′-Pentahydroxyflavone (quercetin, **1**) is an antioxidative compound found in many plants.[1] Antioxidants affecting cellular redox control may be used as powerful tools for the prevention of skeletal muscle atrophy,[2] as oxidative stress is suggested to be involved in the disability of muscle functions.[3] Unloading occurring in immobilization and weightlessness causes disused muscle atrophy. It has been reported that two ubiquitin ligases, atrogin-1 and muscle-specific ring finger protein 1(MuRF-1), are critical in the development of disused muscle atrophy. The aim of the present study was to evaluate the suppressive effect of quercetin (**1**) on ubiquitin ligase-induced muscle atrophy. Quercetin (**1**) mainly exists in the glycoside form in plants. During the absorption process, quercetin glycosides are hydrolyzed to the aglycone (**1**) by epithelial hydrolytic enzymes or enterobacteria in the intestinal tract and metabolized to its glucuronide and/or sulfate conjugates.[4−7] Nevertheless, quercetin (**1**) is found in the skeletal muscle of rats and pigs after the intake of a quercetin-containing diet.[8] Glucuronide conjugates of quercetin are also capable of being converted to the aglycone form by $\beta$-glucuronidase activity.[9] Therefore it is considered meaningful to investigate the effect of quercetin (**1**) in skeletal muscle.




Quercetin (**1**) or flavone (**2**) was injected into the gastrocnemius muscle of one leg of seven-week-old male C57BL/6 mice. Vehicle or *N*-acetyl-L-cysteine was also injected into the gastrocnemius muscle of another leg in the same mouse. After 24 h, half of the mice were subjected to tail suspension (tail-suspension group), while the other half did not undergo tail suspension (ground group). The tail suspension was maintained for 10 days, and the compound injections continued at 24 h intervals. Gastrocnemius muscles were removed on day 10.

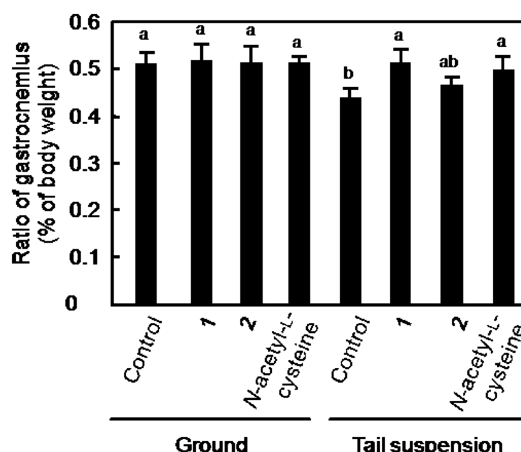



**Figure 1.** Effect of quercetin (**1**) on the weight of mouse gastrocnemius muscle with or without tail suspension. Data are expressed as ratio of gastrocnemious (a percentage of the body weight) and are means ± SE ($n = 4$). Different letters indicate statistically significant differences ($p < 0.05$).

The tail-suspension group had a significantly decreased ratio of gastrocnemius muscle weight:whole-body weight as compared with the ground group (Figure 1). Quercetin (**1**) and *N*-acetyl-L-cysteine suppressed the decrease of muscle weight ratio in the tail-suspension group, whereas flavone (**2**) exhibited no effect. Next, it was examined if quercetin (**1**) suppressed the mRNA expression of atrogin-1 and MuRF-1 in the gastrocnemius muscle using the real-time PCR method (Figure 2A and B). The tail suspension induced mRNA expression of atrogin-1 and MuRF-1, and **1** significantly suppressed the induction of atrogin-1 and MuRF-1 in the tail-suspension group, whereas **2** did not. *N*-Acetyl-L-cysteine suppressed the induction of atrogin-1, but not the induction of MuRF-1 (Figure 2A and B). The concentration of thiobarbituric acid-reactive substances (TBARS) in the gastrocnemius muscle was measured as the index of lipid peroxidation in muscle (Figure 3). The TBARS level in the tail-suspension group was about 1.6 times higher than that of the ground group. Quercetin (**1**) and *N*-acetyl-L-cysteine significantly inhibited enhancement of the level of TBARS in the tail-suspension group, whereas flavone (**2**) showed no inhibition.

These results demonstrate that intramuscular injection of quercetin (**1**) can exert a suppressive effect on disused muscle atrophy associated with the induction of the ubiquitin ligases atrogin-1 and MuRF-1 in mice undergoing tail suspension. It is therefore likely that quercetin (**1**) has the potential to prevent the degradation of

* To whom correspondence should be addressed. Tel: +81 88-633-7087. Fax: +81 88-633-7089. E-mail: terao@nutr.med.tokushima-u.ac.jp.
[†] Department of Food Science.
[‡] Department of Nutritional Physiology.
[§] Department of Clinical Nutrition.

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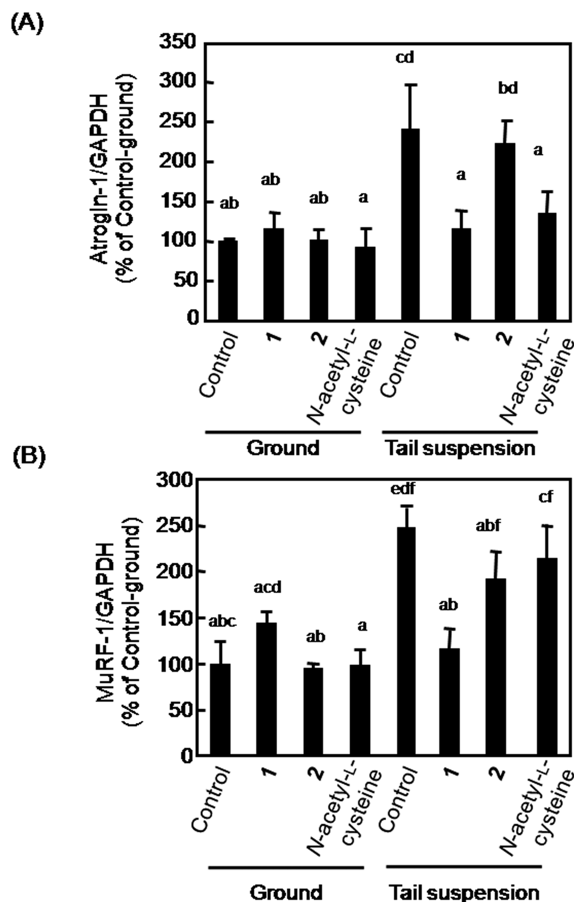


**Figure 2.** Effect of quercetin (**1**) on the expression of atrogin-1 and MuRF-1 in the mouse gastrocnemius muscle with or without tail suspension. mRNA expression level was determined with real-time PCR. Data are shown as a percentage of mRNA level (A: atrogin-1, B: MuRF-1) in control-ground group and are means ± SE ($n$ = 4). Different letters indicate statistically significant differences ($p < 0.05$).

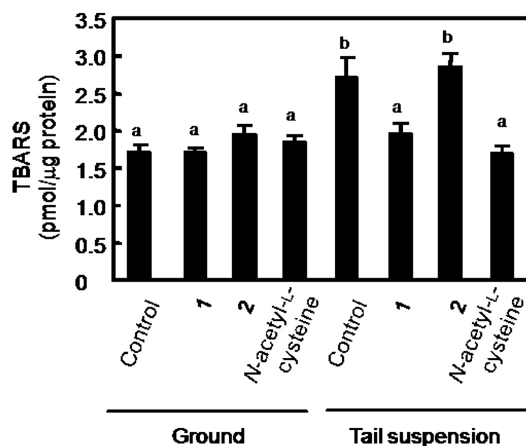


**Figure 3.** Effect of quercetin (**1**) on TBARS levels in the mouse gastrocnemius muscle with or without tail suspension. TBARS concentrations in the gastrocnemius muscle were measured, and the data obtained are means ± SE ($n$ = 4). Different letters indicate statistically significant differences ($p < 0.05$).

skeletal-muscle proteins, resulting in the maintenance of gastrocnemius muscle mass. This is the first report that an antioxidant flavonoid, quercetin (**1**), suppresses disused muscle atrophy in vivo. Although *N*-acetyl-L-cysteine also suppresses the decrease of muscle weight ratio in the tail-suspension group (Figure 1), it did not inhibit MuRF-1 expression in mice that underwent tail suspension (Figure 2). However, **1** and *N*-acetyl-L-cysteine suppressed the loss of gastrocnemius muscle mass in mice that underwent tail suspension (Figure 1). Accordingly, the mechanism of action for **1** may be (at least in part) different from that for *N*-acetyl-L-cysteine. Expression of MuRF-1 and atrogin-1 is regulated by forkhead box O (FOXO) transcription factors,[10] and these promote atrophy in skeletal muscle.[10] Quercetin (**1**) may modulate a signal transduction pathway regulating FOXO function, as flavonoids are known to acts as signaling molecules.[11] Oxidative stress is frequently associated with the degradation of skeletal muscle tissue.[12] It is well known that nitric oxide synthase (NOS) expression is increased during muscle wasting.[13] Activities of antioxidant enzymes involving superoxide dismutase, catalase, and glutathione peroxidase are reduced in atrophy muscle.[14] Our group has found that supplementation of the antioxidative nutrient cysteine prevented unloading-induced ubiquitination in association with redox regulation in rat skeletal muscle.[2] In experiments using cultured myotubes, **1** suppressed the expression of atrogin-1 and MuRF-1 and the MEK/ERK signaling, a well-known mitogen-activated protein kinase (MAPK) pathway to mediate oxidative stress.[15] It is therefore reasonable to propose that the suppressive effect of **1** on the induction of ubiquitin ligase originates from its antioxidative characteristics. The nonantioxidative flavonoid flavone (**2**) did not show any effect on muscle-weight loss or the expression of ubiquitin ligases. The level of lipid peroxidation in the gastrocnemius muscle was expressed by the level of TBARS and was found to be enhanced by tail suspension in accordance with the induction of ubiquitin ligases (Figures 2 and 3). The fact that quercetin (**1**), but not flavone (**2**), inhibited lipid peroxidation supports the idea that **1** attenuates unloading-derived oxidative stress, resulting in suppression of the induction of ubiquitin ligase. The inhibition of lipid peroxidation by *N*-acetyl-L-cysteine, in addition to the suppression of disused muscle atrophy, also indicates that antioxidants capable of regulating cellular oxidative stress are helpful in the prevention of disused muscle atrophy. In conclusion, quercetin (**1**) has the potential to suppress the atrophy of skeletal muscle when concentrated in the target tissue.

## Experimental Section

**General Experimental Procedures.** Seven-week-old male C57BL/6 mice (Japan SLC, Shizuoka, Japan) were housed in a room maintained at 23 ± 1 °C on a 12 h light/dark cycle. They were allowed free access to laboratory diet (AIN-93M; Oriental Yeast Co., Tokyo, Japan) and water. All experimental protocols in this study were approved by the guidelines for the care and use of laboratory animals of the University of Tokushima, Graduate School, Institute of Health Biosciences.

**Tail-Suspension Study.** Compound **1** (purity 98%, Sigma-Aldrich, St. Louis, MO) was mixed with physiological (0.9%) saline containing 0.1% propylene glycol. This mixture was injected into the gastrocnemius muscle (50 μL; 2.5 pmol of quercetin (**1**)/leg) of one leg. Vehicle (50 μL/leg) was also injected into the gastrocnemius muscle of another leg in the same mouse. Flavone (**2**: purity 98%, Wako Pure Chemical Industries, Ltd., Osaka, Japan) or *N*-acetyl-L-cysteine (purity 98%, Wako) was also mixed with the vehicle and injected into the gastrocnemius muscle in a similar manner (2.5 pmol/leg) as above. After 24 h, half of the mice were subjected to tail suspension.[16] Their tails were suspended to keep their rear legs off the ground (tail-suspension group). The other half did not undergo tail suspension (ground group). Tail suspension was maintained for 10 days, and the intramuscular injection of each compound continued at 24 h intervals during the tail suspension. During the tail suspension, the forefoot of the mice touched the ground. These mice were killed 6 h after injection on day 10, and the gastrocnemius muscle was immediately removed. Body weight variations of the mice were not different among each experimental group.

**Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** Total RNA from the muscle was extracted using an Isogen instrument (Nippon Gene, Tokyo, Japan). One microgram of RNA was reverse-transcribed to cDNA using the M-MLB reverse transcriptase kit (Promega, Madison, WI) according to the manufacturer's protocol. To measure the mRNA amount in muscle samples, RT-PCR was

programmed with SYBR green dye using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA) as previously described.[17] The oligonucleotide primers used in this study were 5′-ACCCAGAAGACTGTGGATGG-3′ (S) and 5′-TTCAGCTCTGG-GATGACCTT-3′ (AS) for GAPDH, 5′-GGCGGACGGCTGGAA-3′ (S) and 5′-CAGATTCTCCTTACTGTATACCTCCTTGT-3′ (AS) for Atrogin-1, and 5′-ACGAGAAGAAGAGCGAGCTG-3′ (S) and 5′-CTTGGCACTTGAGAGGAAGG-3′ (AS) for MuRF-1.

**Measurement of Thiobarbituric Acid-Reactive Substances (TBARS).** The muscle samples were homogenized with nine times the volume of phosphate-buffered saline (PBS) buffer and centrifuged at 800*g* for 10 min at 4 °C. Protein concentration in the supernatant was determined by the Bradford method[18] using bovine serum albumin as a standard protein. The supernatant (0.14 mL) was added to 0.05 mL of 10 mM butyl hydroxytoluene in ethanol and 0.5 mL of thiobarbituric acid (TBA) solution (0.25 N HCl containing 1.5% (w/v) trichloroacetic acid (TCA) and 0.375% (w/v) TBA). The mixture was then heated on a boiling water bath for 15 min. After cooling, 1 mL of *n*-butanol was added and the butanol layer obtained by centrifugation. Fluorescence ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 530$ nm) in the butanol layer was measured. The level of TBARS was calculated using 1,1,3,3-tetraethoxypropane as a standard.

**Statistical Analysis.** Data are shown as mean ± SE ($n = 4$). Statistical analysis was performed using two-way ANOVA with the Student−Newman−Keuls multiple comparisons test. *p*-Values below 0.05 were considered significant.

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