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Preventive effect of dietary quercetin on disuse muscle atrophy by targeting mitochondria in denervated mice

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Abstract

Quercetin is a major dietary flavonoid in fruits and vegetables. We aimed to clarify the preventive effect of dietary quercetin on disuse muscle atrophy and the underlying mechanisms. We established a mouse denervation model by cutting the sciatic nerve in the right leg (SNX surgery) to lack of mobilization in hind-limb. Preintake of a quercetin-mixed diet for 14 days before SNX surgery prevented loss of muscle mass and atrophy of muscle fibers in the gastrocnemius muscle (GM). Phosphorylation of Akt, a key phosphorylation pathway of suppression of protein degradation, was activated in the quercetin-mixed diet group with and without SNX surgery. Intake of a quercetin-mixed diet suppressed the generation of hydrogen peroxide originating from mitochondria and elevated mitochondrial peroxisome proliferator-activated receptor-γ coactivator 1α mRNA expression as well as NADH dehydrogenase 4 expression in the GM with SNX surgery. Quercetin and its conjugated metabolites reduced hydrogen peroxide production in the mitochondrial fraction obtained from atrophied muscle. In C2C12 myotubes, quercetin reached the mitochondrial fraction. These findings suggest that dietary quercetin can prevent disuse muscle atrophy by targeting mitochondria in skeletal muscle tissue through protecting mitochondria from decreased biogenesis and reducing mitochondrial hydrogen peroxide release, which can be related to decreased hydrogen peroxide production and/or improvements on antioxidant capacity of mitochondria.

Keywords: Polyphenol; Oxidative stress; Quercetin; Antioxidant; Mitochondria; Disuse muscle atrophy

1. Introduction

Skeletal muscle accounts for approximately 40% of body weight and plays an important role in amino acid and glucose metabolism, protein storage and locomotion. Therefore, the maintenance of muscle mass and its function is essential for human health. Immobilization, such as fixing with a cast, space flight or a bedridden condition, triggers disuse muscle atrophy [1,2]; in these cases, atrophy is caused by an imbalance between protein synthesis and its degradation in the skeletal muscle cells. It has been suggested that the PI3K/Akt phosphorylation pathway, which contributes to protein synthesis, is repressed whereas proteolytic systems are activated in atrophied muscles [3,4]. In ubiquitin-proteasome system, one of the major proteolytic systems in skeletal muscle, ubiquitin ligases such as muscle RING-finger protein-1 (MuRF-1) is transiently up-regulated in the early stage of atrophy. The generation of reactive oxygen species (ROS) is accompanied by these events during disuse muscle atrophy [5]. In fact, 8-hydroxy-2′-deoxyguanosine and proteins conjugated with 4-hydroxy-2-noneral were identified in atrophied muscle of unloaded mice as a result of oxidative modification of DNA and proteins, respectively [6]. Lipid hydroperoxides, primary products of lipid peroxidation, were also detected in the skeletal muscle of unloaded mice [7,8]. Bhattacharya et al. [9] demonstrated that mice with denervated immobilization exhibited an accumulation of nonesterified fatty acid hydroperoxides in the mitochondria of skeletal muscle cells, provoking mitochondrial dysfunction along

Abbreviations: BHT, 2,6-bis(1,1-dimethylethyl)-4-methylphenol; DPPP, diphenyl-1-prenylphosphine; GM, gastrocnemius muscle; MuRF-1, muscle RING-finger protein-1; ND1, NADH dehydrogenase subunit 1; ND4, NADH dehydrogenase subunit 4; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; ROS, reactive oxygen species; SNX, denervation of sciatic nerve; sol, soleus muscle; TA, tibialis anterior.

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with elevated ROS production. Because mitochondrial-produced ROS activates proteolytic systems *via* the redox-sensitive signaling pathway responsible for activation of transcriptional factors, such as NF-κB [10], mitochondria seem to be one of the therapeutic target sites for disuse muscle atrophy. Indeed, in mice with ankle fixation, a small cell-permeable peptide with antioxidant activity could prevent disuse muscle atrophy by targeting mitochondria [6]. In contrast, overexpression of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), one of the indexes of mitochondria biogenesis, prevents muscle atrophy of hindlimb-unloaded mice [11].

Quercetin (3,3′,4′,5,7′-pentahydroxyflavone) is one of the plant flavonoids ubiquitously present in fruits and vegetables. Onions are a major source of quercetin in the human diet [12]. The average quercetin intake in daily diet has been estimated as 9.3 mg per day [13]. Quercetin exists as either aglycone or glycosides, especially glucosides, in plants. However, glucosides undergo hydrolysis in epithelial cells of the small intestine. During absorption, quercetin aglycone is further converted into a metabolites, such as glucuronide (*e.g.*, quercetin 3-*O*-β-glucuronide: Q3GA), sulfate (*e.g.*, quercetin 3′-*O*-sulfate: Q3'S) and/or their *O*-methyl derivatives (isorhamnetin and tamarixetin) [14], and these metabolites circulate in the blood stream and/or the lymphatic system [15]. Quercetin aglycone and/or its metabolites distribute to several tissues, including skeletal muscle [15,16]. However, subcellular localization of quercetin and its metabolites in skeletal muscle cells has not been investigated, although confocal laser scanning microscope analysis was successfully applied to visualize localization in hepatic, endothelial and intestinal cells [17]. *In vitro*, *in vivo* and clinical studies have revealed that quercetin can modulate cellular redox status [18]. We previously reported that intramuscular injection of quercetin suppressed muscle atrophy, which correlated with inhibition of lipid peroxidation in tail-suspension model mice [19]. In addition, dietary quercetin prevents oxidative damages in skeletal muscle [20,21]. An *in vitro* study showed that ferric iron ion with hydrogen-peroxide-induced oxidative damage in myofibrillar proteins was effectively retarded by quercetin [22]. Although these findings suggest that quercetin exerts an antioxidant effect in skeletal muscle, it has never been demonstrated that dietary quercetin is effective in preventing disuse muscle atrophy. Nevertheless, a rodent study revealed that dietary supplementation of quercetin induces expression of PGC-1α, Sirt-1 (a PGC-1α activator) and mitochondrial DNA in the muscle and brain [23]. Thus, it is likely that dietary quercetin attenuates oxidative stress derived from muscle atrophy, resulting in maintenance of mitochondria function in skeletal muscle cells. However, it should be determined whether dietary quercetin helps prevent disuse muscle atrophy by targeting mitochondria biogenesis and/or acting as an antioxidant against ROS release from damaged mitochondria.

The aim of the present study was to determine the potential of dietary quercetin as a mitochondria-targeted food factor to prevent disuse muscle atrophy. In denervated mice, sciatic nerve denervation induces disuse muscle atrophy, including the gastrocnemius (GM) and tibialis anterior (TA) muscles [24,25]. In the atrophied muscle, damaged mitochondria produce hydrogen peroxide [26]. Therefore, the role of quercetin as a mitochondrial antioxidant will be discussed by measuring the modulating effect against hydrogen-peroxide-induced cellular oxidative stress. Our results will be helpful in the evaluation of dietary quercetin for future nutritional therapy on human health.

2. Materials and methods

2.1. Materials

Quercetin and Q3GA were obtained from Nacalai Tesque (Kyoto, Japan) and Extrasynthese (Genay Cedex, France), respectively. Q3'S was synthesized and purified by our research team according to previous reports [27,28].

2.2. Animal experiments

All animal experiments received permission from the Committee on Animal Experiments of Tokushima University (permit number: 11,013) and were performed according to the guidelines for the care and use of laboratory animals set by the University (Tokushima, Japan). The experiments were performed according to previous studies [24]. C57/BL6 mice (7-week-old male, Japan SLC, Shizuoka, Japan) were maintained in a room at 23 ± 1°C on a 12-h light–dark cycle. Quercetin (0.2%, w/w) was mixed with a commercial diet (no flavonoids were detected in the diet: AIN-93 M; Oriental Yeast Company, Tokyo, Japan). Cellulose content was reduced to adjust for the composition of other nutrients. Quercetin-mixed diet or control diet was given to mice for 1 day or 14 days (1-day preintake or 14-day preintake.) The sciatic nerve in the right leg of each mouse was cut (denervation) to induce immobilization (*i.e.*, SNX), which resulted in disuse muscle atrophy in the GM, soleus muscle (sol) and TA. Sham surgery was undertaken in the left leg of each mouse to obtain control muscle. During the development of disuse muscle atrophy, supplementation of a quercetin-mixed diet was continued until the end of the experiment. Plasma was collected at 0 day (with alive) and 4 days after SNX surgery (*i.e.*, day 14 or 18). The GM and sol were collected, and their weights were measured at 4 or 6 days after SNX surgery (*i.e.*, day 18 or 20, respectively). The change in muscle mass was calculated as the weight ratio of SNX muscle to sham muscle. The cross-sectional area of muscle fiber in GM was also determined by hematoxylin–eosin staining according to a previous study [29]. For the measurement of hydrogen peroxide using the Amplex Red method [9], GM was applied for the experiment immediately. For the other experiments, muscles were stored at −80°C until further use.

2.3. Determination of quercetin and its O-methylated metabolites in the GM by high-performance liquid chromatography (HPLC) analysis

Whole GM from SNX muscle or sham muscle was used to detect quercetin and its *O*-methylated metabolites. Sample preparation, including deconjugation, was performed according to a previous report [24]. An HPLC electrochemical detection system (700 mV; Coulochem III, ESA, Cambridge, MA, USA) was used for measurements. A Cadenza 3-μm CD-C18 HPLC column (150×4.6 mm; Imtakt, Portland, OR, USA) was equipped for analysis, and flow rate was 1.0 ml/min. The mobile phase composition was 0.5% phosphoric acid in methanol/0.5% phosphoric acid (44/56, v/v). Quercetin was determined by reference to its respective standard curve, and *O*-methylated metabolites were determined using the standard curve for isorhamnetin.

2.4. Western blot analysis

Western blot analysis was performed according to the method described in a previous study [24]. Briefly, the GM was homogenized with lysis buffer using a protease inhibitor tablet (Complete, Roche, Basel, Switzerland) and a phosphatase inhibitor tablet (PhosSTOP, Roche). After centrifugation, supernatants were collected as whole protein lysates. Anti-pAKTser473 antibody (Cell Signaling Technology, Danvers, MA, USA), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Cell Signaling Technology), anti-Akt antibody (Cell Signaling Technology) and anti-Murf-1 antibody (sc-32920, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were incubated with the membrane for 1 h at room temperature and then incubated with peroxidase-labeled secondary antibody for 1 h. Immunocomplexes on the membrane were visualized using a Chemiluminescence Detection Kit (Amersham ECL Prime, GE Healthcare, Buckinghamshire, U.K.) and were analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA).

2.5. Measurement of insulin-like growth factor 1 (IGF-1) in plasma

We applied plasma sample to Mouse/Rat IGF-I Quantikine ELISA Kit supplied by R&D Systems, Inc. (Minneapolis, MN, USA). We performed the experiment according to manufacture protocol.

2.6. Determination of lipid hydroperoxides in GM

Whole GM at day 22 (8 days after denervation) was homogenized in 1.5 ml phosphate-buffered saline with 50 mM ascorbic acid (pH 7.4). Homogenates were mixed with 1 ml of 1 mM 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) in chloroform and 2 ml water. The chloroform layer was separated by centrifugation (1660 *g*, 5 min, 4°C) to obtain the lipid fraction. The chloroform layer was evaporated with nitrogen gas. The residue was dissolved in a mixture of chloroform containing 1 mM BHT and methanol (1:2, v/v). To detect lipid hydroperoxides, 10 mM diphenyl-1-prenylphosphine (DPPP) (Do Jindo Laboratories, Kumamoto, Japan) was added to the samples for 30 min at 60°C, and then fluorescence (Ex 352 nm, Em 380 nm) was measured [30].

2.7. Measurement of hydrogen peroxide in the mitochondria; fraction of GM

2.7.1. **Experiment I** *(in vivo)*

Sham and SNX GM muscles were collected from quercetin-mixed diet or control diet mice. The mitochondrial fraction was isolated according to a previous report [31]