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Quercetin inhibits adipogenesis of muscle progenitor cells *in vitro*

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ABSTRACT

Muscle satellite cells are committed myogenic progenitors capable of contributing to myogenesis to maintain adult muscle mass and function. Several experiments have demonstrated that muscle satellite cells can differentiate into adipocytes *in vitro*, supporting the mesenchymal differentiation potential of these cells. Moreover, muscle satellite cells may be a source of ectopic muscle adipocytes, explaining the lipid accumulation often observed in aged skeletal muscle (sarcopenia) and in muscles of patients` with diabetes. Quercetin, a polyphenol, is one of the most abundant flavonoids distributed in edible plants, such as onions and apples, and possesses antioxidant, anticancer, and anti-inflammatory properties. In this study, we examined whether quercetin inhibited the adipogenesis of muscle satellite cells *in vitro* with primary cells from rat limbs by culture in the presence of quercetin under adipogenic conditions. Morphological observations, Oil Red-O staining results, triglyceride content analysis, and quantitative reverse transcription polymerase chain reaction revealed that quercetin was capable of inhibiting the adipogenic induction of muscle satellite cells into adipocytes in a dose-dependent manner by suppressing the transcript levels of adipogenic markers, such as peroxisome proliferator-activated receptor-γ and fatty acid binding protein 4. Our results suggested that quercetin inhibited the adipogenesis of muscle satellite cells *in vitro* by suppressing the transcription of adipogenic markers.

1. Introduction

Skeletal muscle has many vital functions, including movement and postural support. Healthy adult muscle is a highly plastic, dynamic tissue capable of responding to physiological stimuli for regeneration and hypertrophy. Muscle satellite cells (mSCs) are major components of resident stem cells that are involved in the regeneration and maintenance of muscle mass and function in adult muscle [1,2]. Named for their anatomical position in muscles, mSCs are located beneath the basal lamina in a dominant quiescent state and express Pax7, which is a member of the paired box (PAX) family of transcription factors. Activated mSCs progress through the cell cycle and express MyoD, a myogenic transcriptional factor, after which they become myoblasts. Most proliferating myoblasts downregulate Pax7 and enter the final differentiation phase through myogenin upregulation, followed by fusion with each other or with existing myotubes.

mSCs are also multipotent and can form adipocyte-like cells, osteocytes, and nerve cells [3,4]. During adipocyte formation, the expression levels of several transcription factors and their target molecules related to adipogenesis, such as peroxisome proliferator-activated receptor-γ (PPAR-γ) and fatty acid binding protein 4 (FABP4), are increased. Muscle disuse, atrophy, and aging increase, whereas muscle mass and function decrease; these features are sometimes accompanied by ectopic lipid deposition and fat mass accumulation [5–9]. Intramuscular lipids have also been observed in injured mutant mice with genetic depletion of Pax7 [10], indicating that defects in mSC function during muscle regeneration lead to ectopic lipid accumulation. Considering these observations, it is possible that dysfunctional mSCs may be a source of ectopic adipocytes, leading to lipid accumulation in impaired regeneration or aged muscles.

Quercetin (3,5,7,3′,4′-pentahydroxyflavone), a polyphenol, is one of the most abundant flavonoids in the human diet and is known to possess antioxidant, anti-inflammatory, and anticancer properties [11,12]. Importantly, quercetin has been shown to have antioxidative effects in muscles or myoblasts, including upregulation of mitochondrial activity and suppression of atrophic factors, in several rodent and *in vitro*

Abbreviations: mSCs, muscle satellite cells; PPAR-γ, peroxisome proliferator-activated receptor gamma; FABP4, fatty acid binding protein 4; TG, triglyceride

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models [13–16]. Moreover, quercetin has been reported to suppress adipocyte differentiation in 3T3-L1 pre-adipocytes [17,18]. Thus, we hypothesized that quercetin may regulate the differentiation of multipotent mSCs into adipocytes.

In the present study, to test this hypothesis, we examined the effects of quercetin in an *in vitro* system. Our findings demonstrated that quercetin inhibited the adipogenesis of mSCs and affected the transcription of *PPAR-γ* and *FABP4*.

2. Material and methods

2.1. Animals

Male F344 rats (10–14 weeks of age; Japan SLC, Inc., Hamamatsu, Shizuoka, Japan) were housed at 23 ± 1 °C under a 12-h light/dark cycle and provided with standard chow and water *ad libitum*. This study was approved by the Juntendo University Animal Care and Use Committee (H26-05) and was performed according to the guiding principles for the care and use of laboratory animals established by the Physiological Society of Japan.

2.2. Satellite cell isolation, culture, and differentiation

Primary mSCs were isolated from the major hindlimb muscles (gastrocnemius, soleus, plantaris, tibialis anterior, extensor digitorum longus, and quadriceps) of rats, as previously described [19], and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. After 20–24 h, unattached cells were collected by centrifugation at 1,500 × *g* for 3 min, and the cell pellet was resuspended in growth medium, *i.e.,* F-10 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal bovine serum and penicillin/streptomycin (Gibco). After cell counting, the cells were seeded on collagen-coated eight-well culture slides (Falcon, Corning, Bedford, MA, USA). After 3 days of incubation, the growth medium was changed to adipogenic induction medium (Lonza, Basel, Switzerland) to induce adipogenesis. After 3 more days of incubation, the adipogenic induction medium was changed to adipogenic maintenance medium (Lonza), followed by incubation for 3–4 days. Quercetin (quercetin dihydrate, Nacalai Tesque, Kyoto, Japan) was added to the cells in the adipogenic induction medium and adipogenic maintenance medium at the indicated concentrations. As negative control, dimethylsulfoxide without quercetin was used.

To confirm the myogenic potency of the isolated cells, the isolated cells were seeded at twice the typical density. After culture in growth medium for 2 days, the cells were shifted to low-serum medium (2% house serum in Dulbecco's modified Eagle's medium) or adipogenic maintenance medium without adipogenic induction medium for 1–6 days to induce myogenesis.

2.3. Immunostaining

To confirm the purity of the isolated cells, after 40 h of culture in growth medium, cells were fixed in methanol at 4 °C for 10 min for immunostaining. After washing, fixed cells were incubated in 10% normal goat serum, 2% bovine serum albumin (BSA), and 0.1% Triton-X100 in phosphate-buffered saline (PBS) for 30 min at room temperature and then further incubated with the following primary antibodies: anti-MyoD (M3512, Dako, Glostrup, Denmark), anti-desmin (D3, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-myogenin (F5D, Developmental Studies Hybridoma Bank), and anti-Pax7 (PAX7, Developmental Studies Hybridoma Bank), diluted with 2% BSA in PBS at 4 °C overnight. After washing with cold PBS four times, cells were incubated with anti-mouse biotinylated secondary antibodies or anti-mouse IgG conjugated with horseradish peroxidase (GE Healthcare, Waukesha, WI, USA) and washed with PBS. To visualize stained cells, we used the peroxidase substrate kits VIP (Vector Laboratories, Burlingame, CA, USA) combined with a VECSTAtin Elite ABC Kit (Vector Laboratories) or ImmPACT DAB (Vector Laboratories). Cells were dehydrated and mounted with Multi Mount 480 (Matsunami Glass, Osaka, Japan). Images of immunostained sections were captured as described below.

Immunofluorescence was performed with anti-myosin heavy chain antibodies (MF20, Developmental Studies Hybridoma Bank) and anti-mouse IgG Alexa Fluor 488-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA), followed by counter staining with 4′,6-diamidino-2-phenylindole (DAPI). Images of immunofluorescence were captured using a microscope (BZ-8000, Keyence, Osaka, Japan) with a 20×/0.75 Plan Apo objective (Olympus, Tokyo, Japan).

2.4. Oil Red-O staining and imaging

Oil Red-O (Sigma-Aldrich, St. Louis, MO, USA) stock solution, at a concentration of 30% (w/v) in isopropanol, was diluted with distilled water (3:5) and used within 2 h of filtration. Cells were fixed in 10% formalin for 10 min and stained with Oil Red-O working solutions for 30 min at room temperature. Cells were then counterstained with hematoxylin (Muto Pure Chemicals, Tokyo, Japan) and mounted with an aqueous mounting medium (Aquatex, 108562, Merck, Darmstadt, Germany). Bright-field images of live and Oil Red-O-stained cells were captured using a microscope (BZ-8000, Keyence) with 2×/0.1 Plan Apo and 20×/0.75 Plan Apo objectives (Olympus).

2.5. Triglyceride (TG) and protein quantification

Cells were washed twice with PBS and harvested in 5% Triton X-100 in PBS. Whole-cell lysates were heated at 90 °C for 5 min and chilled on ice for 3 min, followed by heating and chilling again. After centrifugation at 13,000 rpm for 10 min at 4 °C, the obtained supernatants were used to determine TG and protein concentrations using a LabAssay Triglyceride kit (Wako Pure Chemical, Osaka, Japan) and BCA assay reagents (Thermo Fisher Scientific), respectively.

2.6. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells using an RNeasy Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Isolated RNA was reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA). To measure mRNA levels, real-time PCR was performed with TaqMan Gene Expression Assays using an ABI 7900 real-time system (Life Technologies). All primers and probes were purchased as TaqMan Gene Expression Assays: PPAR-γ (Rn00440945_m1), FABP4 (Rn04219585_m1), MyoD (Rn01457527_g1), and Pax7 (Rn01518732_m1). The relative gene expression levels of each sample were determined using the comparative Ct method. Expression assays for each gene were normalized to 18SrRNA (Hs99999901_s1) and are expressed as fold change relative to the control level.

2.7. Statistical analysis

All values are expressed as means ± standard errors (SEs). Data were analyzed using one-way analysis of variance with Dunnett's *t*-test. Differences with p values of less than 0.05 were considered statistically significant. All statistical analyses were performed using PASW v18 (IBM, Armonk, NY, USA).

3. Results

To determine the characteristics of the cellular phenotype, the myogenic markers MyoD, myogenin, and desmin and the satellite cell-