

The preventive effect of β -carotene on denervation-induced soleus muscle atrophy in mice

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Abstract

Muscle atrophy increases the production of reactive oxygen species and the expression of atrophy-related genes, which are involved in the ubiquitin–proteasome system. In the present study, we investigated the effects of β -carotene on oxidative stress ($100 \mu\text{M-H}_2\text{O}_2$)-induced muscle atrophy in murine C2C12 myotubes. β -Carotene ($10 \mu\text{M}$) restored the H_2O_2 -induced decreased levels of myosin heavy chain and tropomyosin ($P < 0.05$, n 3) and decreased the H_2O_2 -induced increased levels of ubiquitin conjugates. β -Carotene reduced the H_2O_2 -induced increased expression levels of E3 ubiquitin ligases (Atrogin-1 and MuRF1) and deubiquitinating enzymes (USP14 and USP19) ($P < 0.05$, n 3) and attenuated the H_2O_2 -induced nuclear localisation of FOXO3a. Furthermore, we determined the effects of β -carotene on denervation-induced muscle atrophy. Male ddY mice (8 weeks old, n 30) were divided into two groups and orally pre-administered micelle with or without β -carotene (0.5 mg once daily) for 2 weeks, followed by denervation in the right hindlimb. β -Carotene was further administered once daily until the end of the experiment. At day 3 after denervation, the ratio of soleus muscle mass in the denervated leg to that in the sham leg was significantly higher in β -carotene-administered mice than in control vehicle-administered ones ($P < 0.05$, n 5). In the denervated soleus muscle, β -carotene administration significantly decreased the expression levels of Atrogin-1, MuRF1, USP14 and USP19 ($P < 0.05$, n 5) and the levels of ubiquitin conjugates. These results indicate that β -carotene attenuates soleus muscle loss, perhaps by repressing the expressions of Atrogin-1, MuRF1, USP14 and USP19, at the early stage of soleus muscle atrophy.

Key words: β -Carotene: Muscle atrophy: Ubiquitin ligase: Deubiquitinating enzymes: Denervation

Skeletal muscle is the most abundant tissue in the human body. The functions of skeletal muscle are glucose and lipid metabolism in addition to mobility and movement. A prolonged period of skeletal muscle disuse due to denervation, hindlimb unloading, immobilisation or bed rest leads to muscle atrophy, thereby decreasing the mass and function of skeletal muscle⁽¹⁾. A potential factor in the loss of muscle mass is oxidative stress. The production of reactive oxygen species increases in muscle mitochondria during denervation, hindlimb unloading, and sarcopenia^(2,3). Antioxidants are expected to attenuate the muscle disuse-induced increase in reactive oxygen species emission and to prevent skeletal muscle from oxidative stress^(1,4,5). However, such antioxidants have not yet been identified.

The balance between the synthesis and degradation of muscle proteins such as myosin heavy chain (MyHC) and

tropomyosin regulates muscle mass, and increased protein degradation results in a loss of muscle mass. Muscle atrophy increases the expression of atrophy-related genes (atrogenes) such as muscle-specific E3 ubiquitin ligases (*Atrogin-1* and *MuRF1*)^(6–9). Okamoto *et al.*⁽⁷⁾ and Sacheck *et al.*⁽⁸⁾ reported that the expressions of *Atrogin-1* and *MuRF1* are dramatically increased during the early stage or early phase (e.g. at 3 d) of disuse atrophy induced by immobilisation and denervation, respectively. The ubiquitin–proteasome system controls the degradation of aberrant proteins. *Atrogin-1* and *MuRF1* conjugates ubiquitin to muscle proteins, and subsequently the conjugated proteins are degraded by the proteasome. Because *Atrogin-1* and *MuRF1* knockout mice are resistant to muscle atrophy⁽⁶⁾, the down-regulation of *Atrogin-1* and *MuRF1* expressions should contribute to the prevention of muscle atrophy. Many atrogenes are induced by the FOXO family of

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DUB, deubiquitinating enzyme; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MyHC, myosin heavy chain; TBARS, thiobarbituric acid-reactive substances; USP, ubiquitin-specific peptidase.

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transcription factors⁽¹⁰⁾. Oxidative stress is involved in the activation of FOXO3a, and activated FOXO3a induces the expression of *Atrogin-1* and *MuRF1*^(10–13). On the other hand, the ubiquitin–proteasome system is also regulated by the deubiquitination reaction, in which deubiquitinating enzymes (DUB) cleave ubiquitin from ubiquitin-conjugated proteins and recycle free ubiquitin⁽¹⁴⁾. DUB are called ubiquitin-specific peptidases (USP). Muscle atrophy increases the expression of *USP14* and *USP19* mRNA, and knockdown of *USP19* inhibits muscle atrophy^(15–17). Therefore, the expression levels of ubiquitin ligases and DUB should be monitored during muscle atrophy.

Edible plant-derived antioxidants such as carotenoids provide some protection against oxidative stress. β -Carotene is a dietary source of vitamin A⁽¹⁸⁾ and has been shown to have beneficial effects *in vitro* as an antioxidant^(19–22). However, it remains unclear whether dietary supplementation of β -carotene attenuates disuse-induced muscle atrophy. Here, we report that oxidative stress increases *USP14* and *USP19* expressions in addition to *Atrogin-1* and *MuRF1* and that β -carotene inhibits their increased gene expression *in vitro*. Furthermore, we demonstrate that oral administration of β -carotene to mice attenuates denervation-induced loss of soleus muscle mass and inhibits increases in the expressions of two ubiquitin ligases and two DUB at the early stage of disuse atrophy.

Experimental methods

Cell culture

Murine skeletal muscle C2C12 myoblasts were obtained from the RIKEN Cell Bank (Ibaraki, Japan). C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units penicillin/ml, and 100 μ g streptomycin/ml (growth medium) at 37°C in a 5% CO₂–95% air atmosphere at 100% humidity. To induce differentiation from myoblasts to myotubes, C2C12 myoblasts were grown to 90% confluency in the growth medium and shifted to DMEM supplemented with 2% horse serum (differentiation medium). The differentiation medium was replaced at 48 h intervals for 8 d. When cells were incubated in the presence of β -carotene, β -carotene was dissolved in dimethyl sulfoxide. As a vehicle control, dimethyl sulfoxide was added at a final concentration of 0.1%.

Animals and β -carotene administration

The care of all animals in the present study was in accordance with the guidelines of the Animal Care and Use Committee of Osaka Prefecture University. A total of thirty male Kwl:ddY mice (7 weeks old) were obtained from Kiwa Laboratory Animals (Wakayama, Japan). These mice had free access to water and a non-purified diet (CE-2; Clea Japan, Inc.) and were kept at a controlled temperature (23 \pm 2°C), humidity (60 \pm 10%) and lighting (a 12 h light–12 h dark cycle starting at 08.00 hours). After 1 week of adaptation (8 weeks old), mice (30–35 g body weight) were

randomly divided into two groups (*n* 15 per group). One group was orally administered micellar β -carotene (0.5 mg once daily) by direct intubation to the stomach (β -carotene group) and the other was orally administered micelles without β -carotene as a vehicle (vehicle group) for 2 weeks. Micellar β -carotene was prepared as mixed micelles as described previously⁽²³⁾. In brief, β -carotene (5 mg) and sodium taurocholate (12.9 mg) were mixed in ethanol (0.5 ml) and dried under N₂ gas. The residue was mixed in lysophosphatidyl choline (1.31 mg), oleic acid (4.23 mg) and mono-olein (1.78 mg) in distilled water (2 ml) under shading.

Denervation of hindlimb and tissue harvest

A 4 to 5 mm segment of the sciatic nerve in the right hindlimb of 10-week-old mice (*n* 10 per group) was removed under pentobarbital (50 mg/kg body weight, intraperitoneal) anaesthesia, and the contralateral non-denervated left hindlimb was sham-operated as an internal control. β -Carotene was further administered (0.5 mg once daily) until the end of the experiment. Food intake in the experiment was controlled because the non-denervated hindlimb from the same animal was used as a control. The animals in each group were killed at 3 and 7 d (*n* 5 per time point) after denervation under pentobarbital (50 mg/kg body weight intraperitoneal) anaesthesia (days 3 and 7, respectively). On the other hand, ten 10-week-old mice (*n* 5 per group) were killed without denervation (day 0). The skeletal muscle tissues (soleus and gastrocnemius muscles) were excised, cleaned off extraneous tissues and weighed. The sample was frozen in liquid N₂ and stored at –80°C until use.

RNA isolation and RT-PCR analysis

C2C12 myotubes were pre-incubated in the presence of vehicle or β -carotene for 12 h, followed by rinsing twice with PBS, pH 7.4, and incubation in fresh DMEM differentiation medium containing 100 μ M-H₂O₂ for 3 h. Total RNA was isolated using Sepasol-RNA II Super G (Nacalai Tesque) according to the manufacturer's instructions, and reverse-transcribed. On the other hand, total RNA from skeletal muscle was extracted from frozen tissues. The resultant complementary DNA were subjected to quantitative real-time PCR using the following specific primers: *Atrogin-1* (forward primer 5'-TCACAGCTCACATCCCTGAG-3' and reverse primer 5'-GACT-TGCCGACTCTCTGGAC-3'); *MuRF1* (forward primer 5'-GACT-CCTGCAGAGTGACCAAG-3' and reverse primer 5'-CTTCTAC-AATGCTCTTGATGAGC-3'); *USP14* (forward primer 5'-CCAA-GCCACTTGTGCATCTTCA-3' and reverse primer 5'-TAGATAA-GGCAGAGGACGGGCA-3'). Specific primers for *USP19*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β -actin were as described previously⁽²⁴⁾. The PCR profiles consisted of denaturation at 95°C for 1 min, primer annealing at 57°C for 1 min and primer extension at 72°C for 30 s. The final primer extension was performed at 72°C for 10 min. The PCR in quantitative real-time PCR was performed with the Plexor One-Step qRT-PCR System (Promega) on a Thermal Cycler Dice, TP-800 (Takara). C_t values were transformed into

relative quantification data by the $2^{-\Delta\Delta C_t}$ method, and were normalised to the C_t values of β -actin or GAPDH.

Western blotting

C2C12 myotubes were pre-incubated in the presence of vehicle or β-carotene for 12 h and rinsed twice with PBS, followed by incubation in fresh DMEM differentiation medium containing 100 μM-H₂O₂. To accumulate ubiquitin-conjugated proteins, myotubes were incubated in the presence of 10 μM-MG132. After 12 h, C2C12 myotubes were lysed in lysis buffer A (50 mM-Tris-HCl, pH 7.5, containing 150 mM-NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM-EDTA, 1 mM-4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg leupeptin/ml and 1 μg aprotinin/ml) and sonicated. On the other hand, soleus and gastrocnemius muscles (5 mg) were homogenised in lysis buffer B (20 mM-HEPES-NaOH, pH 7.5, containing 0.5% Nonidet P-40, 1 mM-EDTA, 50 μM-butylated hydroxytoluene, 1 mM-4-(2-aminoethyl) benzenesulfonyl fluoride, 10 μg leupeptin/ml and 1 μg aprotinin/ml). Lysates were subjected to SDS-PAGE and analysed by Western blotting with the following primary antibodies: rabbit polyclonal anti-GAPDH (1:5000)⁽²⁵⁾, and mouse monoclonal anti-MyHC (1:2000) (MF-20, supernatant; Developmental Studies Hybridoma Bank), anti-tropomyosin (1:2000) (CH1, supernatant; Developmental Studies Hybridoma Bank), and anti-ubiquitin antibodies (1:5000) (FK2; Nippon Biotest Laboratories, Inc.). Immunoreactive proteins

were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000) or anti-mouse IgG (1:3000) and reacted with the Super Signal West Femto Chemiluminescent Substrate (Pierce Biotechnology) or Immobilon Western Chemiluminescent HRP Substrate (Millipore), followed by detection with the LAS4000 imaging system (GE Healthcare). The intensities of immunoreactive proteins were quantified by densitometry using Image J (version 1.44; National Institutes of Health).

Immunofluorescence analysis

C2C12 myotubes were pre-incubated in the presence of vehicle or β-carotene for 12 h and rinsed twice with PBS, followed by incubation in fresh DMEM differentiation medium containing 100 μM-H₂O₂ for 3 h. C2C12 myotubes were fixed in 4% paraformaldehyde in PBS for 10 min and permeabilised with 0.2% Triton X-100 in PBS for 5 min as described previously⁽²⁶⁾. The cells were incubated with rabbit monoclonal anti-FOXO3a antibody (1:500) (75D8; Cell Signaling Technology) at 4°C overnight, followed by incubation with anti-rabbit Alexa 488-conjugated antibody (1:2000) (Cell Signaling Technology) at room temperature under shading. After 1 h, the cells were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (1 μg/ml) in PBS for 10 min. Negative controls were treated with only a secondary antibody. Fluorescent images were analysed by using a BIOREVO BZ-9000 fluorescence microscope (Keyence).

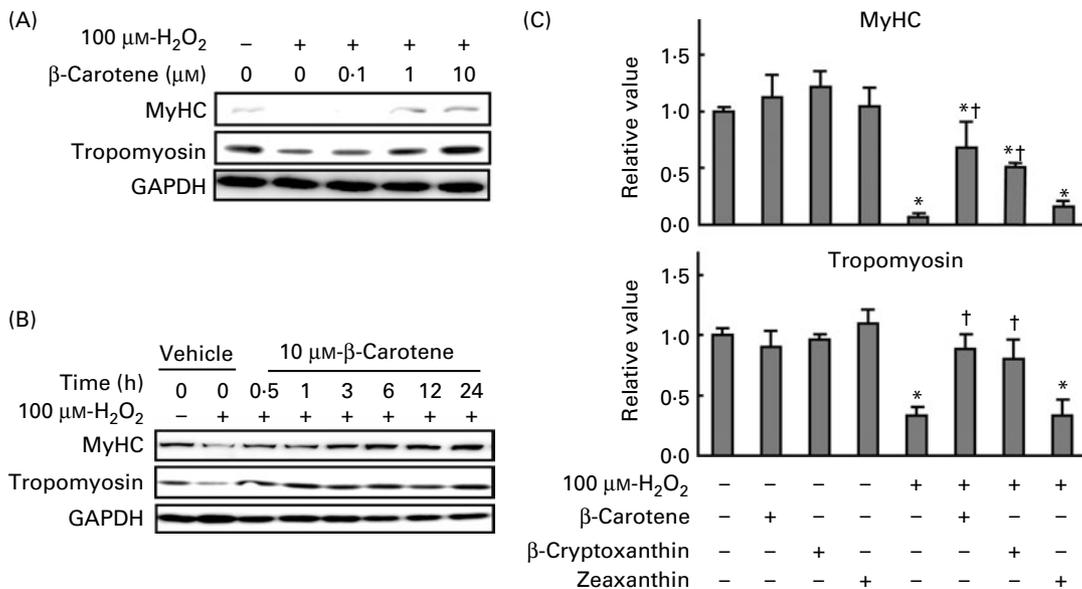


Fig. 1. Inhibitory effect of β-carotene on hydrogen peroxide-induced C2C12 myotube atrophy. (A) C2C12 myotubes were pre-incubated in the presence of various concentrations (0–10 μM) of β-carotene for 12 h, and further incubated in fresh medium containing 100 μM-hydrogen peroxide for 12 h. Western blot analyses with anti-myosin heavy chain (MyHC), anti-tropomyosin and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies are shown. (B) C2C12 myotubes were pre-incubated in the presence of vehicle or 10 μM-β-carotene for various time periods, and further incubated in fresh medium containing 100 μM-hydrogen peroxide for 12 h. Western blot analyses with anti-MyHC, anti-tropomyosin and anti-GAPDH antibodies are shown. (C) C2C12 myotubes were pre-incubated in the presence of vehicle or 10 μM-carotenoid (β-carotene, β-cryptoxanthin or zeaxanthin) for 12 h, and further incubated in fresh medium containing 100 μM-hydrogen peroxide for 12 h. Western blot analyses with anti-MyHC, anti-tropomyosin and anti-GAPDH antibodies were performed, and the intensities of immunoreactive bands were quantified (n 3). The levels of MyHC and tropomyosin were normalised to the level of GAPDH, and data were expressed as a relative value of the intensity of the control (hydrogen peroxide-untreated myotubes in the presence of vehicle). Values are means, with their standard deviations represented by vertical bars. Mean value was significantly different compared with hydrogen peroxide-untreated myotubes under the same carotenoid treatment conditions: * P< 0.05. Mean value was significantly different compared with vehicle-treated myotubes under hydrogen peroxide treatment conditions: † P< 0.05.

Thiobarbituric acid-reactive substances assay

Lipid peroxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS) according to the method of Ohkawa *et al.*⁽²⁷⁾. Soleus and gastrocnemius muscles were homogenised in lysis buffer B (20 mM-HEPES–NaOH, pH 7.5, containing 0.5% Nonidet P-40, 1 mM-EDTA, 50 μ M-butylated hydroxytoluene, 1 mM-4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μ g leupeptin/ml and 1 μ g aprotinin/ml), and the homogenates were subjected to the TBARS assay. The homogenates were incubated with 0.8% 2-thiobarbituric acid for 45 min at 90°C. The organic layer was extracted with *n*-butanol, and fluorescence was measured at an excitation of 544 nm and an emission of 590 nm (Fluoroskan Ascent FL; Labsystems). The values are expressed in nmol malonaldehyde/mg protein.

Statistics

Data for RT-PCR analysis in C2C12 myotubes were analysed by one-way ANOVA with Tukey's *post hoc* test, and the others were analysed by two-way ANOVA with Bonferroni's *post hoc* test using GraphPad Prism 4 (GraphPad Software). Data are expressed as means and standard deviations, and differences were considered as statistically significant at $P < 0.05$.

Results

β -Carotene inhibits hydrogen peroxide-induced muscle atrophy in C2C12 myotubes

We determined the effects of β -carotene on muscle atrophy induced by oxidative stress in C2C12 myotubes. β -Carotene dose-dependently attenuated H_2O_2 -induced decreases in MyHC and tropomyosin expression (Fig. 1(A)). When C2C12 myotubes were pre-incubated in the presence of β -carotene for various time periods (0.5–24 h), followed by treatment with H_2O_2 , β -carotene time-dependently abolished H_2O_2 -induced decreases in MyHC and tropomyosin expression (Fig. 1(B)). Furthermore, the effects of other carotenoids on H_2O_2 -induced muscle atrophy were determined in C2C12 cells. As with β -carotene, β -cryptoxanthin and zeaxanthin inhibited H_2O_2 -induced decreases in MyHC and tropomyosin expression in the order β -carotene = β -cryptoxanthin > zeaxanthin (Fig. 1(C)).

β -Carotene represses hydrogen peroxide-induced levels of ubiquitin ligases and deubiquitinating enzymes in C2C12 myotubes

β -Carotene repressed the levels of ubiquitin conjugates in a dose-dependent manner in H_2O_2 -treated myotubes (Fig. 2). β -Carotene dose-dependently inhibited the H_2O_2 -induced increased expressions of the two ubiquitin ligases (Fig. 3(A) and (B)). When C2C12 myotubes were exposed to H_2O_2 , the mRNA levels of *USP14* and *USP19* were increased. When myotubes were pre-incubated with β -carotene before exposure to H_2O_2 , β -carotene dose-dependently inhibited

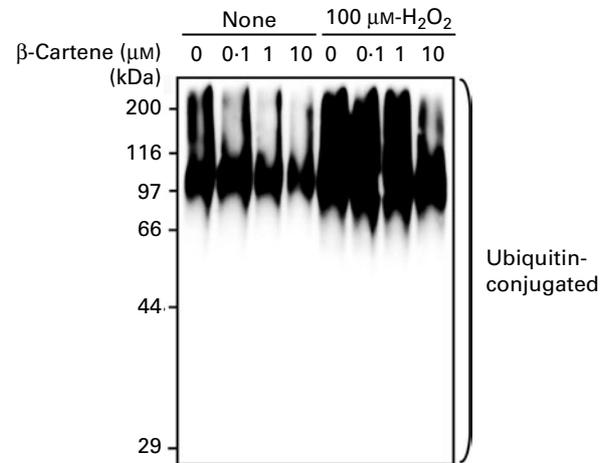


Fig. 2. Effects of β -carotene on the levels of ubiquitin conjugates in hydrogen peroxide-treated C2C12 myotubes. C2C12 myotubes were pre-incubated in the presence of various concentrations (0–10 μ M) of β -carotene for 12 h, and further incubated in fresh medium containing 100 μ M-hydrogen peroxide in the presence of 10 μ M-MG132 for 12 h. Western blot analysis with anti-ubiquitin antibody is shown.

H_2O_2 -induced increases in *USP14* and *USP19* mRNA expressions (Fig. 3(C) and (D)).

β -Carotene inhibits the nuclear localisation of FOXO3a

FOXO3a was localised throughout the cytoplasm in the presence or absence of β -carotene (Fig. 4, first and second panels from the left side). H_2O_2 treatment induced the nuclear localisation of FOXO3a (Fig. 4, third panels from left side), while β -carotene inhibited it (Fig. 4, first panels from the right side).

β -Carotene inhibits the loss of soleus muscle mass induced by denervation

To assess the effect of β -carotene on denervation-induced muscle atrophy, the experimental protocol was designed as shown in Fig. 5(A). In the soleus muscle, the ratio of muscle mass in the denervated leg to that in the sham leg was significantly higher in the β -carotene group than in the vehicle group at day 3, but not at day 7, after denervation (Fig. 5(B), left panel). On the contrary, there was no significant difference in the ratio of gastrocnemius muscle mass between the vehicle group and the β -carotene group (Fig. 5(B), right panel).

β -Carotene represses thiobarbituric acid-reactive substances levels increased by denervation

In the soleus muscle, denervation increased TBARS levels in the vehicle group, but not in the β -carotene group, at day 3 (Fig. 6(A)). β -Carotene reduced TBARS levels in both sham legs and denervated legs, compared with vehicle. In contrast, in the gastrocnemius muscle, denervation had no influence

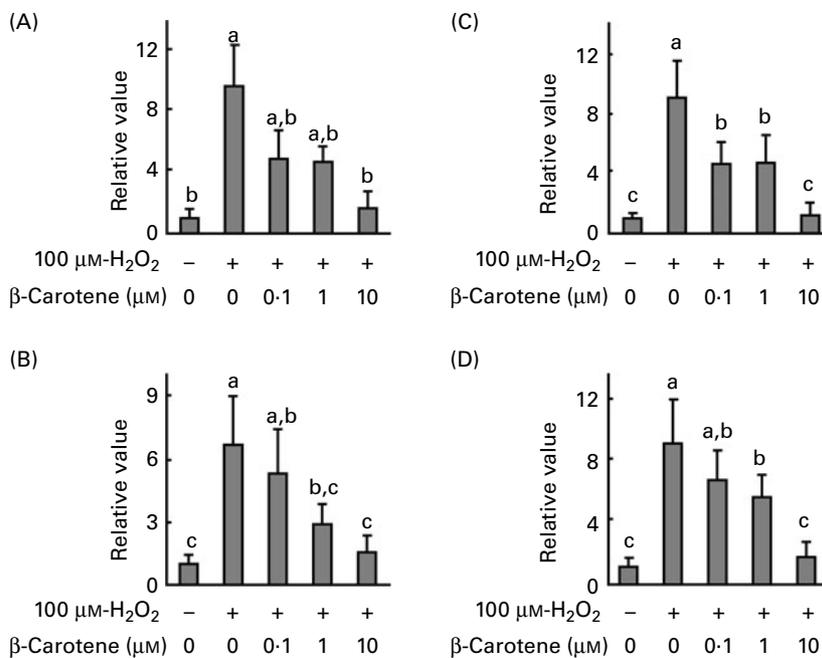


Fig. 3. Effects of β-carotene on ubiquitin ligases and deubiquitinating enzyme expressions in hydrogen peroxide-treated C2C12 myotubes. C2C12 myotubes were pre-incubated in the presence of various concentrations (0–10 μM) of β-carotene for 12 h, and further incubated in fresh medium containing 100 μM-hydrogen peroxide for 3 h. Quantitative real-time PCR were performed for (A) *Atrogin-1*, (B) *MuRF1*, (C) *USP14*, (D) *USP19* and *GAPDH* mRNA. Data were normalised to *GAPDH* as the endogenous control. Relative values are means (*n* 3), with their standard deviations represented by vertical bars. ^{a,b,c} Mean values with unlike letters were significantly different (*P*<0.05).

on TBARS levels in the vehicle or β-carotene group, and β-carotene had no influence on TBARS levels in sham legs or denervated legs (Fig. 6(B)).

β-Carotene inhibits the denervation-induced increased levels of ubiquitin conjugates in the soleus muscle

We determined the effects of β-carotene on the levels of ubiquitin conjugates in soleus and gastrocnemius muscles. In soleus and gastrocnemius muscles, the levels of ubiquitin

conjugates were increased by denervation (Fig. 7(A) and (B)). β-Carotene attenuated the denervation-induced accumulation of ubiquitin conjugates in the soleus muscle, but not in the gastrocnemius muscle.

β-Carotene represses the expression of ubiquitin ligases and deubiquitinating enzymes increased by denervation

Denervation significantly increased the levels of *Atrogin-1*, *MuRF1*, *USP14* and *USP19* in the soleus muscle in the vehicle

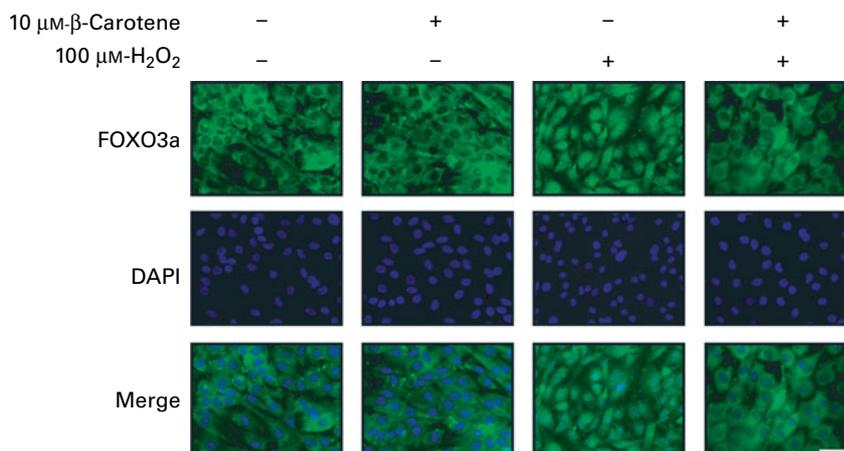


Fig. 4. Inhibitory effect of β-carotene on the nuclear localisation of FOXO3a induced by hydrogen peroxide. C2C12 myotubes were pre-incubated in the presence of vehicle or 10 μM-β-carotene for 12 h, and further incubated in fresh medium containing 100 μM-hydrogen peroxide for 3 h. Immunoreaction with anti-FOXO3a antibody was performed, followed by incubation with fluorescent-labelled secondary antibody. The nuclei were counterstained with 4,4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The scale bar represents 100 μm.

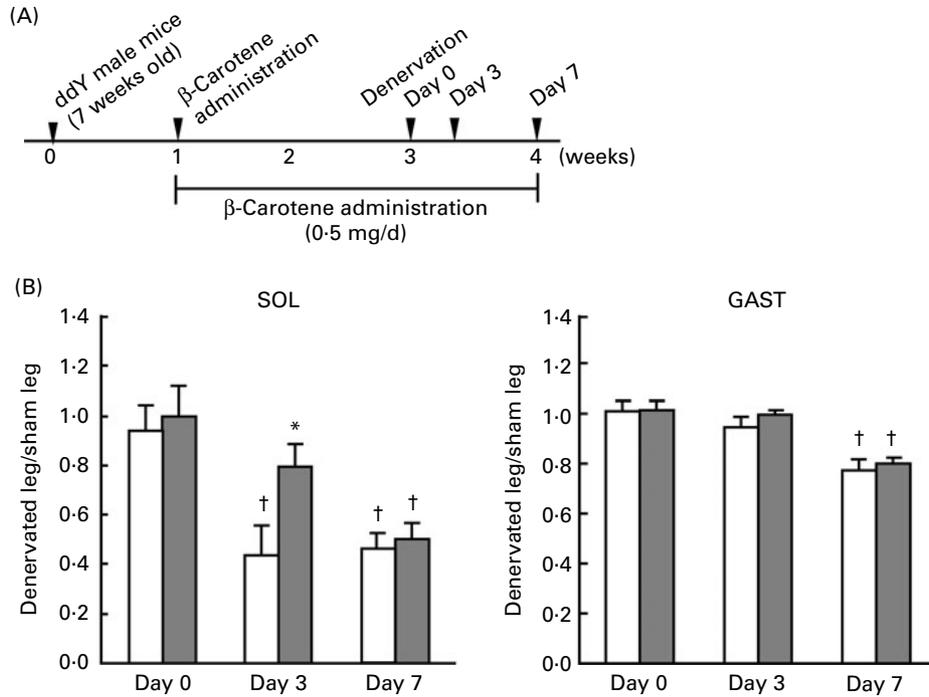


Fig. 5. Effects of β -carotene on denervation-induced skeletal muscle atrophy. (A) Experimental protocol. Micellar β -carotene was orally administered (0.5 mg once daily). After 2 weeks of administration, muscle atrophy due to denervation was induced, and once-daily administration of micellar β -carotene was performed until the end of the experiment (β -carotene group). As a negative control, micelles without β -carotene were administered in place of micellar β -carotene (vehicle group). (B) At days 3 and 7 after denervation, the ratio of soleus muscle (SOL) or gastrocnemius muscle (GAST) mass in the denervated leg to that in the sham leg was determined. Values are means (n 5), with their standard deviations represented by vertical bars. Mean value was significantly different compared with the ratio in the vehicle group at the same time point: * P <0.05. Mean value was significantly different compared with the ratio at day 0 in each group (vehicle group or β -carotene group): $\dagger P$ <0.05. □, Vehicle; ■, β -carotene.

group at day 3 (Fig. 8). In contrast, β -carotene reduced the levels of *Atrogin-1*, *MuRF1*, *USP14* and *USP19* in denervated legs, compared with vehicle.

Discussion

In skeletal muscle, disuse (e.g. denervation, lengthy bed rest or limb immobilisation) causes atrophy accompanied by a

reduction of mass. Muscle atrophy results in dysfunction in muscles. Disuse causes an imbalance between reactive oxygen species production and scavenging, resulting in the accumulation of reactive oxygen species in the mitochondria⁽²⁸⁾. Because oxidative stress seems to be involved in disuse atrophy^(3,4), antioxidants are expected to prevent the progression of muscle atrophy and to improve the muscle mass and function. In the present study, we demonstrated

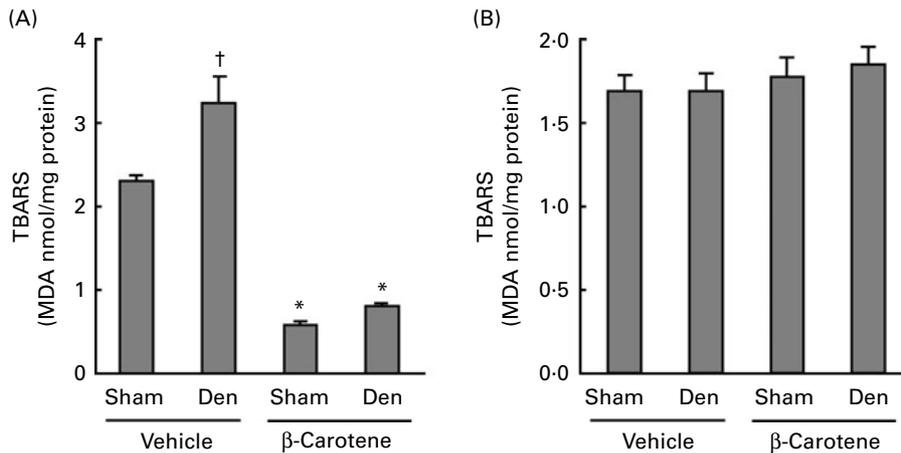


Fig. 6. Effects of β -carotene on thiobarbituric acid-reactive substances (TBARS). Mice were administered micelles with (β -carotene group) or without (vehicle group) β -carotene. (A) Soleus and (B) gastrocnemius muscles of sham legs (Sham) and denervated legs (Den) were homogenised, and TBARS levels were determined. Values are means (expressed in nmol malondialdehyde (MDA)/mg protein), with their standard deviations represented by vertical bars (n 4). Mean value was significantly different compared with the vehicle group in the same legs (Sham or Den): * P <0.05. Mean value was significantly different compared with Sham in the same group (vehicle group or β -carotene group): $\dagger P$ <0.05.

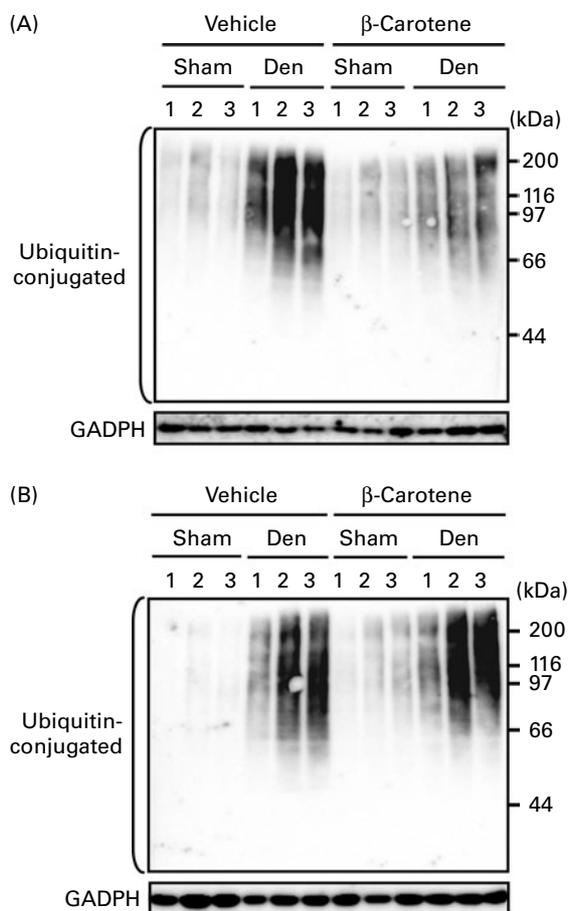


Fig. 7. Effects of β-carotene on the levels of ubiquitin conjugates in denervated skeletal muscles. Mice were administrated micelles with (β-carotene group) or without (vehicle group) β-carotene. (A) Soleus and (B) gastrocnemius muscles were excised from denervated legs (Den) and sham legs (Sham) at day 3 post-denervation (*n* 3). Western blot analyses were performed with anti-ubiquitin and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies.

that β-carotene administration significantly prevents the loss of soleus muscle mass, but not gastrocnemius muscle mass, at day 3 after denervation. However, β-carotene did not affect the masses of soleus and gastrocnemius muscles at day 7 after denervation. Skeletal muscle fibres are classified into two major types: slow twitch and fast twitch. MyHC expresses as slow-type and fast-type isoforms, which are the major proteins responsible for each muscle fibre type. MyHC isoforms work in association with tropomyosin, which is one of the myofibrillar proteins, for muscle contraction. Thus, MyHC and tropomyosin are dominant structural proteins in differentiated muscle and are generally used as markers of myotubes. In the present study, we selected the soleus and gastrocnemius muscles as examples of slow- and fast-twitch muscles, respectively. During muscle disuse atrophy, the mass of slow-twitch soleus muscle is lost more rapidly than that of fast-twitch gastrocnemius muscle^(7,29), and protein oxidation and lipid peroxidation occur in the soleus muscle more than in the gastrocnemius muscle⁽³⁰⁾. These results are consistent with the present results showing

the decreased mass and the increased TBARS level in the soleus muscle, but not in the gastrocnemius muscle, at 3d after denervation. In the present *in vitro* experiments, pre-treatment with β-carotene inhibited the H₂O₂-induced reduction of MyHC and tropomyosin levels (Fig. 1). The inhibitory effects on muscle atrophy were in the order β-carotene = β-cryptoxanthin > zeaxanthin, consistent with a previous report⁽³¹⁾ that the sequence for relative antioxidant activities is β-carotene = β-cryptoxanthin > zeaxanthin. Furthermore, β-carotene inhibited the denervation-induced reduction of muscle mass and the increase in TBARS levels in the soleus muscle at day 3. These results suggest that the antioxidant activity of β-carotene has a beneficial effect on the retardation of soleus muscle loss at the early stage of muscle atrophy (e.g. day 3).

β-Carotene administration attenuated the level of ubiquitin conjugates and the expression levels of *Atrogin-1* and *MuRF1* induced by denervation in the soleus muscle. Vitamin E supplementation partially prevents the increases in *Atrogin-1* and *MuRF1* mRNA levels and the decrease in soleus muscle mass induced by unloading and immobilisation^(32,33). Furthermore, β-carotene inhibited the H₂O₂-induced nuclear localisation of FOXO3a. H₂O₂ induces p38 (mitogen-activated protein kinase)- and c-Jun N-terminal kinase-mediated nuclear accumulation of FOXO3a, and nuclear FOXO3a up-regulates the expression of *Atrogin-1* and *MuRF1*^(10,13). Various antioxidants inhibit the activation of p38 induced by mitochondrial oxidative damage^(34,35). These results suggest that β-carotene represses atrophy-induced *Atrogin-1* and *MuRF1* gene expression by inhibiting oxidative stress-activated FOXO3a signalling.

The levels of *USP14* and *USP19* mRNA were increased in H₂O₂-treated C2C12 myotubes, and β-carotene inhibited their increases. Denervation increased the expression levels of *USP14* and *USP19* mRNA in the soleus muscle, whereas β-carotene significantly reduced their increased expressions. DUB also control the levels of ubiquitin conjugates in the ubiquitin–proteasome system, and the expression levels of *USP14* and *USP19* mRNA are increased during muscle atrophy^(15,16). Although the levels of *Atrogin-1* and *MuRF1* are usually measured during muscle atrophy, the levels of DUB such as *USP14* and *USP19* also should be monitored as atrogenes.

The beneficial effects of β-carotene on muscle atrophy were lost on day 7 (Fig. 5(B)). The expressions of *Atrogin-1* and *MuRF1* reach a peak at day 3 (during the early phase) after disuse atrophy due to denervation and then return to basal levels at day 14 after denervation⁽⁸⁾. However, the relative muscle weight to body weight continues to decrease even after 14d post-denervation. These results suggest that the early and later phases of muscle atrophy due to denervation are regulated by at least two distinct mechanisms. β-Carotene seems to have a beneficial effect on muscle atrophy induced by the ubiquitin–proteasome system during the early phase of muscle atrophy (e.g. day 3).

We demonstrated the beneficial effects of β-carotene on H₂O₂-induced muscle atrophy in C2C12 myotubes. When human subjects receive daily administration of β-carotene

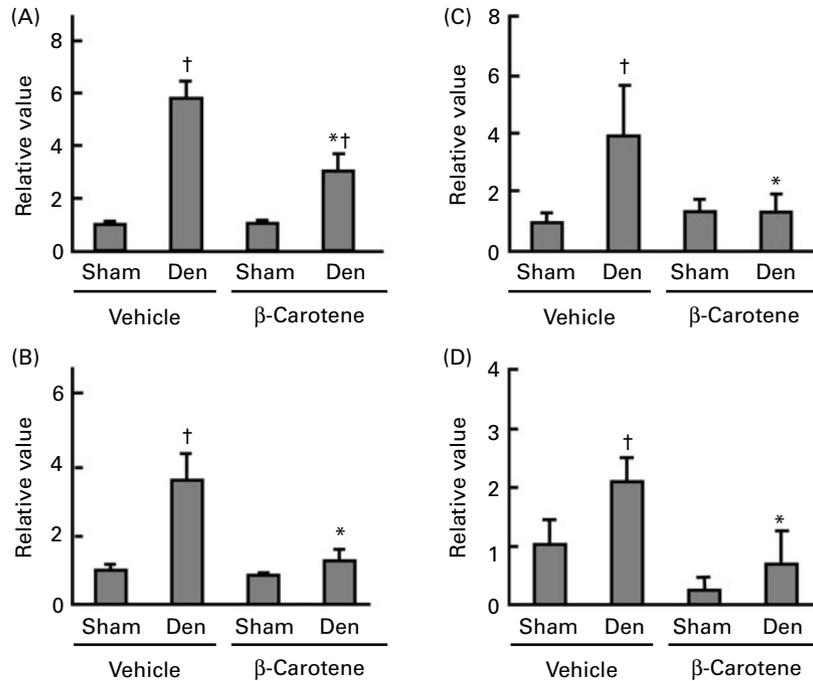


Fig. 8. Effects of β -carotene on ubiquitin ligase and deubiquitinating enzyme expressions in the denervated soleus muscle. Mice were administrated micelles with (β -carotene group) or without (vehicle group) β -carotene. Total RNA was prepared from the soleus muscle in denervated legs (Den) and sham legs (Sham) at day 3 after denervation. (A) *Atrogin-1*, (B) *MuRF1*, (C) *USP14*, (D) *USP19* and β -actin mRNA levels were analysed by quantitative real-time PCR. Data were normalised to β -actin. Values are means (n 5), with their standard deviations represented by vertical bars. Mean value was significantly different compared with the vehicle group in the legs with the same treatment (Sham or Den): * $P < 0.05$. Mean value was significantly different compared with Sham in the same group (vehicle group or β -carotene group): † $P < 0.05$.

(45 mg) for 30 d, plasma concentrations of β -carotene are increased within 2–4 d after administration and then remain elevated⁽³⁶⁾. Although there are inter-individual differences in the extent of the increase in β -carotene concentrations in plasma, β -carotene concentrations are increased to approximately 5 μM in some subjects. On the other hand, plasma concentrations of β -carotene after β -carotene supplementation (20 mg/d) for 3 years reach up to 5.6 μM in human subjects⁽³⁷⁾. Therefore, we investigated the effects of β -carotene at physiologically achievable concentrations (0.1–10 μM) on oxidative stress-induced muscle atrophy in C2C12 myotubes. Although most of the carrots (*Daucus carota* L.) contain β -carotene at > 10 mg/100 g dry weight^(38,39), humans would need to take β -carotene as a supplement rather than as a regular diet to increase plasma β -carotene concentrations to 5–10 μM .

Absorption efficiency of carotenoids in rodents is generally poorer than that in humans⁽⁴⁰⁾. However, absorption efficiency of β -carotene is enhanced in rodents when β -carotene is solubilised in phospholipid-mixed micelles⁽⁴¹⁾. In OUTB-Wistar rats (42 g body weight), the plasma concentration of β -carotene is approximately 0.2 μM at 1 h after oral administration of β -carotene (approximately 20 μg) in micelles. These results suggest that approximately 15 μg β -carotene in micelles is required for ddY mice (30–35 g body weight) to increase plasma β -carotene concentrations to 0.2 μM . Furthermore, because absorption of β -carotene is increased in a dose-dependent manner⁽⁴²⁾, we predicted that administration of a 25- to 50-fold excess of β -carotene (0.375–0.750 mg)

would be required to increase plasma β -carotene concentration to 5–10 μM . We are attempting to determine the pharmacokinetic properties of β -carotene in plasma in mice administered 0.5 mg β -carotene.

We have demonstrated that dietary supplementation of β -carotene is useful as a chemopreventive agent for improvement of disuse-induced muscle mass loss at the early stage of soleus muscle atrophy. Because decreasing the nuclear FOXO3a expression level is effective in repressing the progression of muscle atrophy, it is of interest to find dietary antioxidants that prevent oxidative stress-induced nuclear localisation of FOXO3a. Furthermore, the mechanism by which the expression of DUB is induced by oxidative stress remains to be elucidated.

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