Original Article

Carotenoid transporter CD36 expression depends on hypoxia-inducible factor-1α in mouse soleus muscles

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(Received 10 December, 2021; Accepted 22 March, 2022; Released online in J-STAGE as advance publication 26 May, 2022)

Dietary β-carotene induces muscle hypertrophy and prevents muscle atrophy in red slow-twitch soleus muscles, but not in white fast-twitch extensor digitorum longus (EDL) muscles and gastrocnemius muscles. However, it remains unclear why these beneficial effects of β-carotene are elicited in soleus muscles. To address this issue, we focused on carotenoid transporters in skeletal muscles. In mice, *Cd36* mRNA levels were higher in red muscle than in white muscle. The siRNA-mediated knockdown of CD36 decreased β-carotene uptake in C2C12 myotubes. In soleus muscles, CD36 knockdown inhibited β-carotene-induced increase in muscle mass. Intravenous injection of the hypoxia marker pimonidazole produced more pimonidazole-bound proteins in soleus muscles than in EDL muscles, and the hypoxia-inducible factor-1 (HIF-1) α protein level was higher in soleus muscles than in EDL muscles. In C2C12 myotubes, hypoxia increased the expression of CD36 and HIF-1α at the protein and mRNA levels, and HIF-1α knockdown reduced hypoxia-induced increase in *Cd36* mRNA level. In soleus muscles, HIF-1α knockdown reduced *Cd36* mRNA level. These results indicate that CD36 is predominantly involved in β-carotene-induced increase in soleus muscle mass of mice. Furthermore, we demonstrate that CD36 expression depends on HIF-1α in the soleus muscles of mice, even under normal physiological conditions.

***Key Words*: carotenoid transporter, CD36, β-carotene, hypoxia-inducible factor-1α, soleus muscle**

Skeletal muscle, the most abundant tissue in mammals, has remarkable plasticity and accounts for 30–40% of body weight in humans. Skeletal muscle plays critical roles not only in physical activities, such as postural maintenance and locomotion, but also in the regulation of energy metabolism throughout the body. Reduction in normal physical activities due to trauma or a sedentary lifestyle induces the loss of skeletal muscle masses (muscle atrophy) and increases the risk of developing metabolic disorders, such as obesity and type 2 diabetes.[1] Therefore, the maintenance or enhancement of skeletal muscle masses is a reasonable strategy to prevent a decrease in mobility and the development of metabolic diseases. Recently, the use of specific food components, such as supplements and naturally occurring nutraceuticals, has emerged as a promising approach to maintain and enhance skeletal muscle masses and prevent skeletal muscle atrophy. Identifying transporters that contribute to the uptake of these food components into skeletal muscle and understanding how their expression is regulated are important for maintaining skeletal muscle health.

Carotenoids are fat-soluble pigments that are present in edible plants. Carotenoids are present in human blood and, like other dietary lipids, are taken up into cells via lipid transporters, such as CD36, scavenger receptor class B type I (SR-BI), and Niemann-Pick C1-like 1 (NPC1L1).[2,3] β-Carotene, a major dietary source of provitamin A, is one of the most abundant carotenoids present in circulating human blood and acts as a natural antioxidant. β-Carotene is converted to all-*trans* retinal by β-carotene 15,15'-dioxygenase 1 (BCO1),[4] and all-*trans* retinal is further metabolized to all-*trans* retinoic acid (ATRA). Through extensive studies on this essential nutrient, the physiological roles of ATRA have become increasingly linked to its function as a ligand for retinoic acid receptors (RARs).[5] ATRA binds to RARs bound to retinoic acid response elements (RAREs) in the promoter regions of target genes, resulting in the regulation of expression of these genes.

Skeletal muscle is composed of four major muscle fibers, types I, IIa, IIx, and IIb, based on the presence of specific myosin heavy chain (MyHC) isoforms.[6] Muscle fibers are classified into slow-twitch (type I) and fast-twitch (types IIa, IIx, and IIb). Type I and IIa fibers, also called red muscles, have large numbers of mitochondria and high myoglobin content and produce ATP by oxidative metabolism, allowing for a high level of fatigue resistance and prolonged contraction duration.[7] By contrast, type IIx and IIb fibers, also called white muscles, are not rich in mitochondria or myoglobin, acquire ATP by glycolysis, produce high contraction forces, and have low resistance to fatigue. Soleus muscles express more MyHC I and MyHC IIa isoforms than other MyHC isoforms, and extensor digitorum longus (EDL) muscles express more MyHC IIb isoform.[8] The gastrocnemius muscles are mainly composed of white muscles (white gastrocnemius), whereas a part of the gastrocnemius muscle is composed of red muscles (red gastrocnemius). Red gastrocnemius muscles express more MyHC I and MyHC IIa isoforms, and white gastrocnemius muscles express MyHC IIb isoform.[9] The capillary architectures and tortuosities are different between the soleus and superficial gastrocnemius muscles; the higher number of capillaries and anastomoses, the smaller diameter of capillaries, and the greater tortuosity in soleus muscles.[10] These differences suggest differential oxygen demands between soleus and gastrocnemius muscles.

Hypoxia inducible factor-1 (HIF-1) is the central regulator that drives adaptive responses to low oxygen availability and functions as a primary transcriptional factor for hypoxia-inducible

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doi: 10.3164/jcbn.21-163

genes.[11] HIF-1 binds to the hypoxia response element of target genes and activates their transcription. The target genes of HIF-1 regulate oxygen homeostasis, metabolic states, growth, and differentiation. HIF-1 is a heterodimeric protein consisting of an α subunit (HIF-1α) and a β subunit (HIF-1β/ARNT).[12] Under normoxic conditions, HIF-1α is rapidly hydroxylated by prolyl hydroxylase domain proteins on two proline residues (Pro402 and Pro564) in an oxygen-dependent manner. Subsequently, HIF-1α is ubiquitinated by the E3 ubiquitin ligase von Hippel Lindau and is degraded by the proteasome system. By contrast, under hypoxic conditions, HIF-1α is stably expressed because the O_2-dependent modifications by prolyl hydroxylase domain proteins are inhibited. In skeletal muscles, HIF-1 induces the expression of genes involved in glycolysis during exercise and the loss of HIF-1α results in altered exercise endurance.[13] On the other hand, 3-week exposure to hypoxia (8% O_2) increases MyHC I gene expression in soleus and EDL muscles of young mice (4 weeks old) and in EDL muscles of aged mice (52 weeks old).[14] In C2C12 cells, the knockdown of HIF-1α reduces MyHC I gene expression.[15] Therefore, the microenvironments in slow fibers with more MyHC I might be less oxygenated than those in fast fibers, and HIF-1α stabilization due to hypoxia might contribute to the molecular characterization in slow-twitch fibers.

Dietary β-carotene prevents denervation-induced muscle atrophy in the soleus muscles, but not in the gastrocnemius muscles, of mice.[16] Furthermore, dietary β-carotene increases muscle masses in red muscles (e.g., soleus muscles), but not in white muscles (e.g., EDL, gastrocnemius, and plantaris muscles), of mice.[17] The cross-sectional area in the soleus muscle fibers is increased, indicating that dietary β-carotene induces soleus muscle hypertrophy. In the soleus muscle of the β-carotene-administered mice, twitch force tends to be increased ($p = 0.06$), and tetanic force is increased. Specific force (force for the cross-sectional area) remains unchanged, indicating that β-carotene induces functional hypertrophy in soleus muscle. Thus, β-carotene improves soleus muscle both quantitatively and qualitatively. However, it remains unclear why the beneficial effects of β-carotene are elicited in the red slow-twitch muscles, but not in the white fast-twitch muscles. In the present study, we focused on the expression levels of carotenoid transporters in red and white muscles to elucidate the mechanism by which β-carotene has beneficial effects on soleus muscles. We report that CD36 is a primary carotenoid transporter for β-carotene in soleus muscles. Furthermore, we assessed the involvement of HIF-1α in the regulation of CD36 expression in soleus muscles under normal physiological conditions.

Materials and Methods

Animals. All mice were cared for in accordance with the guideline of the Animal Care and Use Committee of Osaka Prefecture University, which provided ethical approval for the present study (approval no. 29-27). Male C57BL6 mice were obtained from Kiwa Laboratory Animals (Wakayama, Japan) and had free access to water and a standard rodent diet. The mice were housed under controlled temperature (23 ± 2°C), humidity (60 ± 10%), and light (a 12-h light/dark cycle starting at 08:00 a.m.).

Cell culture. Murine myoblast C2C12 cells were obtained from the European Collection of Authenticated Cell Cultures of Public Health England (Porton Down, Salisbury, UK). C2C12 myoblasts were cultured as described previously.[18] Briefly, C2C12 myoblasts were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (termed growth medium) at 37°C under 5% CO_2. To induce differentiation of myoblasts into myotubes, myoblasts were shifted from growth medium to Dulbecco's modified Eagle medium supplemented with 2% horse serum and the above antibiotics (termed differentiation medium) when C2C12 myoblasts were grown to 90% confluency. The differentiation medium was replaced every 48 h. Hypoxia was induced by culturing cells in a multigas incubator with 1% O_2, 5% CO_2, and 94% N_2, as described previously.[19] For the experiments assessing the effects of ATRA and β-carotene, cells were cultured in growth medium and differentiation medium containing 10% dextran-coated charcoal-stripped fetal bovine serum and 2% dextran-coated charcoal-stripped horse serum (termed steroid-free growth medium and steroid-free differentiation medium), respectively.

β-Carotene administration. After 7 days of adaptation, 8-week-old mice were randomly assigned to two groups: one group was orally administered micellar β-carotene (0.5 mg, once daily), and the other was orally administered micelles without β-carotene as a vehicle, both for 14 days. The preparation of micellar β-carotene was described previously.[17] Briefly, β-carotene and sodium taurocholate were dissolved in ethanol and dried under nitrogen. The residue was mixed with lysophosphatidylcholine, oleic acid, and mono-olein in distilled water.

***In vitro* siRNA-mediated knockdown.** Control siRNA (siControl) was purchase from Koken (catalog number S21-25P; Tokyo, Japan). The siRNA-targeting sequences were as follows: siCD36#1, 5'-AAACCCAGAUGACGUGGCAAA-3'; siCD36#2, 5'-CAAAGAGGUCCUUACACAU-3'; siHIF-1α#1, 5'-CAA GCAACUGUCAUAUAUAAUA-3'; and siHIF-1α#2, 5'-GCC GCUCAAUUUAUGAAUA-3'. The duplexes (20 nM) were introduced into C2C12 cells using Lipofectamine RNAiMAX reagent and Opti-MEM (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol.

***In vivo* siRNA-mediated knockdown.** Murine CD36 siRNA (siCD36#1), murine HIF-1α siRNA (siHIF1α#1), and control siRNA (siControl) were transfected into hindlimb muscles according to the protocol described previously.[20] Briefly, siCD36#1, siHIF-1α#1, and siControl solutions were prepared using atelocollagen (10 μM, AteloGene Local Use; Koken). The siCD36 solution or the siHIF-1α solution (50 μl) was injected into the right soleus muscle, and the siControl solution (50 μl) was injected into the left soleus muscle. For the knockdown of CD36, siCD36#1 was injected at days 0, 5, and 10. For the knockdown of HIF-1α, siHIF-1α#1 was injected into the left soleus muscle and soleus muscle was harvested 2 days after injection.

Quantitative PCR analyses. The total RNA was isolated from skeletal muscles and C2C12 myotubes using Sepasol-RNA II Super G (Nacalai Tesque, Kyoto, Japan) and reverse-transcribed using random primers or oligo(dT)20 primer and ReverTraAce (Toyobo, Osaka, Japan). The resultant cDNA was analyzed by quantitative PCR (qPCR) using the following specific primers: *Cd36* (forward primer, 5'-GCCAAGCTA TTGCGACATGA-3'; reverse primer, 5'-CAATGGTTGTCT GGATTCTGG-3'); *Scarb1* (forward primer, 5'-GCCTGTTTG TTGGGATGAA-3'; reverse primer, 5'-ATCTTGCTGAGT CCGTTCC-3'); *Npc1l1* (forward primer, 5'-TGGACTGGA AGGACCATTTCC-3'; reverse primer, 5'-GACAGGTGCCCC GTAGTCA-3'); *Hif1a* (forward primer, 5'-ATAGCTTCGCAG AATGCTCAGA-3'; reverse primer, 5'-CAGTCACCTGGT TGCTGCAA-3'); *Actb* (forward primer, 5'-TTGCTGACAGGA TGCAGAAG-3'; reverse primer, 5'-GTACTTGCGCTCAGG AGGAG-3'); *Myh7* (forward primer, 5'-ACAGAGGAAGAC AGGAAGAACCTAC-3'; reverse primer, 5'-GGGCTTCACAGG CATCCTTAG-3'); *Myh2* (forward primer, 5'-ACTTTGGCACTA CGGGGAAAC-3'; reverse primer, 5'-CAGCAGCATTTCGAT CAGCTC-3'); *Myh4* (forward primer, 5'-CTTTGCTTACGTCAG TCAAGGT-3'; reverse primer, 5'-AGCGCCTGTGAGCTTGTA AA-3'); and *Myh1* (forward primer, 5'-CTCTTCCCGCTTTGG TAAGTT-3'; reverse primer, 5'-CAGGAGCATTTCGATTAG

ATCCG-3'). qPCR was performed using SYBR Premix Ex Taq II DNA polymerase (Takara Bio Inc., Shiga, Japan) and a Thermal Cycler Dice, TP-800 (Takara Bio Inc.). Ct values were transformed into relative quantification data by the $2^{-\Delta\Delta CT}$ method. The Ct values of *Actb* or *18S rRNA* were used as an internal control.

Western blot analysis. Skeletal muscles were homogenized in a lysis buffer (50 mM Tris-HCl, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM DTT, 1 mM Na_3VO_4, 10 mM NaF, 10 mM NaPPi, and 10 mM NaMo) and centrifuged at 20,000 × *g* for 15 min at 4°C. C2C12 myotubes were sonicated in a lysis buffer and centrifuged at 20,000 × *g* for 15 min at 4°C. The supernatants from homogenates and lysates were subjected to SDS-PAGE and analyzed by Western blot analysis using mouse monoclonal antibodies [anti-MyHC I (clone BA-D5; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti-MyHC IIB (clone BF-F3; Developmental Studies Hybridoma Bank, University of Iowa), and anti-β-actin (clone 2D4H5, Proteintech Group, Inc., Chicago, IL) antibodies], and rabbit polyclonal antibodies [anti-HIF-1α (Bethyl Laboratories; Montogomery, TX) and anti-CD36 (Novus Biologicals; Littleton, CO) antibodies]. The immunoreactive proteins were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (BioRad, Hercules, CA) and anti-mouse IgG (BioRad) and reacted with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA), followed by detection with an LAS4000 imaging system (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK).

Pimonidazole injection. Ninety minutes before euthanasia, pimonidazole hydrochloride (Natural Pharmacia International, Inc., Burlington, MA) diluted in saline at a concentration of 30 mg/ml was injected into the tail vein (60 mg/kg body weight). At the end point, skeletal muscle tissues were harvested. Skeletal muscles were homogenized in a lysis buffer and centrifuged at 20,000 × *g* for 15 min at 4°C. The pimonidazole adducts in the supernatants were detected using Hypoxyprobe-1 Plus kit (Hypoxyprobe, Inc., Burlington, MA). In brief, the pimonidazole adducts were analyzed by Western blot analysis using FITC-conjugated mouse monoclonal antibody (clone 4.3.11.3) as a primary antibody and horseradish peroxidase-conjugated rabbit anti-FITC IgG as a secondary antibody, followed by reaction with Immobilon Western Chemiluminescent HRP Substrate.

Reporter assay. The retinoic acid-responsive reporter vector, pRARE-Luc, was constructed by the insertion of an RARE oligonucleotide (5'-CTAGCGAGCAGGTCACCAGGAGG TCACCAGGAGGTCACCAGGAGGTCACCAGGAGGTCAA-3') into the *Nhe* I and *Bgl* II sites of the pGL3-promoter vector. C2C12 myoblasts were grown on 48-well plates in steroid-free growth medium and transiently transfected with pRARE-Luc and pGL4.73[hRluc/SV40] using HilyMax (Dojindo, Kumamoto, Japan) for 24 h. To induce differentiation from myoblasts into myotubes, the medium was shifted to the steroid-free differentiation medium. After 48 h, cells were cultured in the presence of ATRA or β-carotene for 24 h. When CD36 expression was knocked down, cells were transfected with siCD36#1 for 24 h before culture with ATRA or β-carotene. DMSO was used as vehicle. Cells were harvested and lysed, and firefly and *Renilla* luciferase activities were determined with the Dual-Luciferase reporter assay kit and GloMax 20/20 Luminometer (Promega Corp., Madison, WI). Transfection efficiency was normalized using pGL4.73[hRluc/SV40]. Data were expressed as relative light units (RLU, calculated as firefly luciferase activity divided by *Renilla* luciferase activity).

Measurement of intracellular β-carotene. C2C12 myotubes in 35-mm dishes were cultured in differentiation medium in the presence of 10 μM β-carotene for 12 h at 37°C or 4°C as a negative metabolic control. Cells were then washed three times with PBS. Cells were sonicated in 0.1 ml of sucrose buffer (20 mM Hepes-NaOH, pH 7.5, containing 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM DTT) containing 100 pmol of β-cryptoxanthin (an internal standard) and centrifuged at 15,000 × *g* for 15 min. The supernatant was mixed with 0.2 ml of a sucrose buffer. Samples were extracted three times with 0.3 ml of hexane, dried under nitrogen, and reconstituted in the appropriate mobile phase [methanol:acetonitrile:chloroform = 47:47:6 (v/v/v)]. The samples were analyzed by HPLC with LC-2000Plus (JASCO Corporation, Tokyo, Japan) equipped with a UV/visible dual beam detector (UV-2070 Plus). The column was a 4.6 × 250 mm Ultrasphere COSMOSIL Packed Column 5C18-AR-II (Nacalai Tesque). Carotenoids were eluted by an isocratic mobile phase [methanol:acetonitrile:chloroform = 47:47:6 (v/v/v)] at a flow rate of 1 ml/min at 40°C and monitored by measuring absorbance at 450 nm.

Statistical analyses. The comparisons of data between two groups were analyzed by Student's *t* test. The others were analyzed by one-way ANOVA with Dunnett's post hoc test or two-way ANOVA with Tukey's post hoc test using JMP statistical software ver. 8.0.1 (SAS 284 Institute, Cary, NC). Data are expressed as means ± SD. Statistically significant differences were considered at $p<0.05$.

Results

CD36 expression is higher in soleus muscles than in EDL muscles. To assess which carotenoid transporters are expressed in skeletal muscles, the mRNA levels of *Cd36*, *Scarb1*, and *Npc1l1*, encoding CD36, SR-BI, and NPC1L1, respectively, were determined by qPCR. The expression levels of *Cd36* mRNA were higher in soleus muscles than in EDL, tibialis anterior (TA), and plantaris muscles (Fig. 1A). When the gastrocnemius muscles were separated into the red and white regions, *Cd36* mRNA levels were higher in red gastrocnemius muscles than in white gastrocnemius muscles. The levels of *Scarb1* and *Npc1l1* mRNA were not significantly different between the soleus and EDL muscles or between the red and white regions of the gastrocnemius muscles. Soleus muscles and red gastrocnemius muscles expressed more *Myh7* mRNA, a marker for slow-twitch fibers, than did EDL muscles and white gastrocnemius muscles, which expressed more *Myh4* mRNA, a marker for fast-twitch fibers. Furthermore, CD36 protein was also expressed in the soleus muscles and the red gastrocnemius muscles at higher levels than in the EDL muscles and the white gastrocnemius muscles (Fig. 1B). MyHC I expression was higher in soleus muscles and red gastrocnemius muscles than in EDL muscles and white gastrocnemius muscles. These results suggest that among these three carotenoid transporters, CD36 is primarily expressed in soleus muscles and red gastrocnemius muscles.

CD36 is involved in β-carotene uptake into C2C12 myotubes. To determine the effects of *Cd36* depletion on β-carotene uptake in skeletal muscle cells, C2C12 myotubes were transfected with two types of CD36 siRNA (siCD36#1 and siCD36#2), followed by culture with β-carotene. Both CD36 siRNAs resulted in approximately 90% reduction in *Cd36* mRNA levels, but had no influence on *Scarb1* or *Npc1l1* mRNA levels (Fig. 2A). The knockdown of CD36 by siRNA (siCD36#1 and siCD36#2) resulted in approximately 40% decrease in β-carotene uptake in myotubes (Fig. 2B, left panel). Furthermore, we assessed the effects of *Cd36* depletion on β-carotene uptake at 4°C as a negative metabolic control. Culturing cells at 4°C inhibits metabolism, including endocytosis. In control siRNA-transfected cells, the level of β-carotene uptake at 4°C was approximately 60% compared to that at 37°C (Fig. 2B, right panel). Knockdown of CD36 had no influence on β-carotene uptake at 4°C. These results that β-carotene is taken up not only

doi: 10.3164/jcbn.21-163

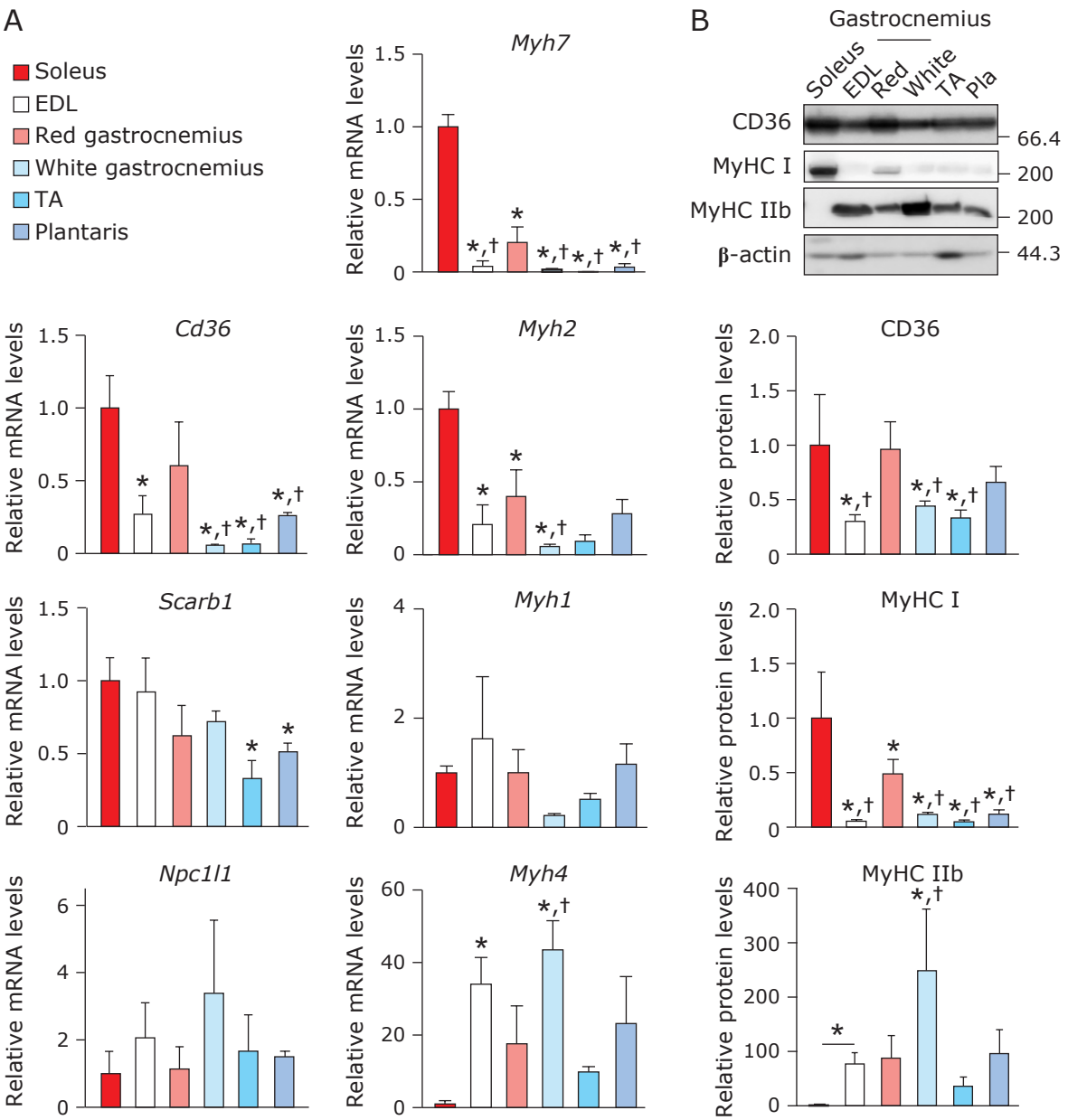

Fig. 1. Expression levels of carotenoid transporters in skeletal muscles. (A) Total RNA was isolated from soleus, EDL, TA, plantaris muscles, and red and white regions of gastrocnemius muscles. The mRNA levels of three carotenoid transporters and MyHC were determined by qPCR and normalized to *Actb* mRNA levels. Data are presented as means ± SD ($n = 3$). (B) Proteins from muscle homogenates were analyzed by Western blot analysis using anti-CD36, anti-β-actin, anti-MyHC I, and anti-MyHC IIb antibodies. Data are presented as means ± SD ($n = 4$). *$p<0.05$ vs soleus muscle, †$p<0.05$ vs red gastrocnemius muscles, by one-way ANOVA with Tukey's post hoc test.

by CD36, but also by passive diffusion that is independent of metabolism. Next, we determined the effects of CD36 depletion on RAR transcriptional activity in the presence of ATRA or β-carotene. RAR transcriptional activities were enhanced in the presence of β-carotene as well as in the presence of ATRA (Fig. 2C). The depletion of CD36 by siCD36#1 inhibited the increase in RAR transcriptional activity in the presence of β-carotene, but had no influence on ATRA-activated RAR transcriptional activity (Fig. 2D). Taken together, these results indicate that CD36 is involved in uptake of β-carotene into C2C12 myotubes.

CD36 is involved in β-carotene-induce increase in soleus muscle masses. To determine the effects of *Cd36* depletion on β-carotene-induced increase in soleus muscle masses, mice were divided into two groups, termed the control and β-carotene groups. Mice in both groups were transfected with control siRNA and CD36 siRNA (siCD36#1) in the left and right soleus muscles, respectively, at days 0, 5, and 10 during the experiment (Fig. 3A). Mice in the control and β-carotene groups were orally administered vehicle and β-carotene, respectively, for 14 days. The injection of CD36 siRNA resulted in a reduction of the *Cd36* mRNA expression level by approximately 55% (Fig. 3B). Dietary β-carotene increased the muscle mass in the control siRNA-injected soleus muscles and had no effects on the muscle mass in the CD36 siRNA-injected soleus muscles (Fig. 3C).

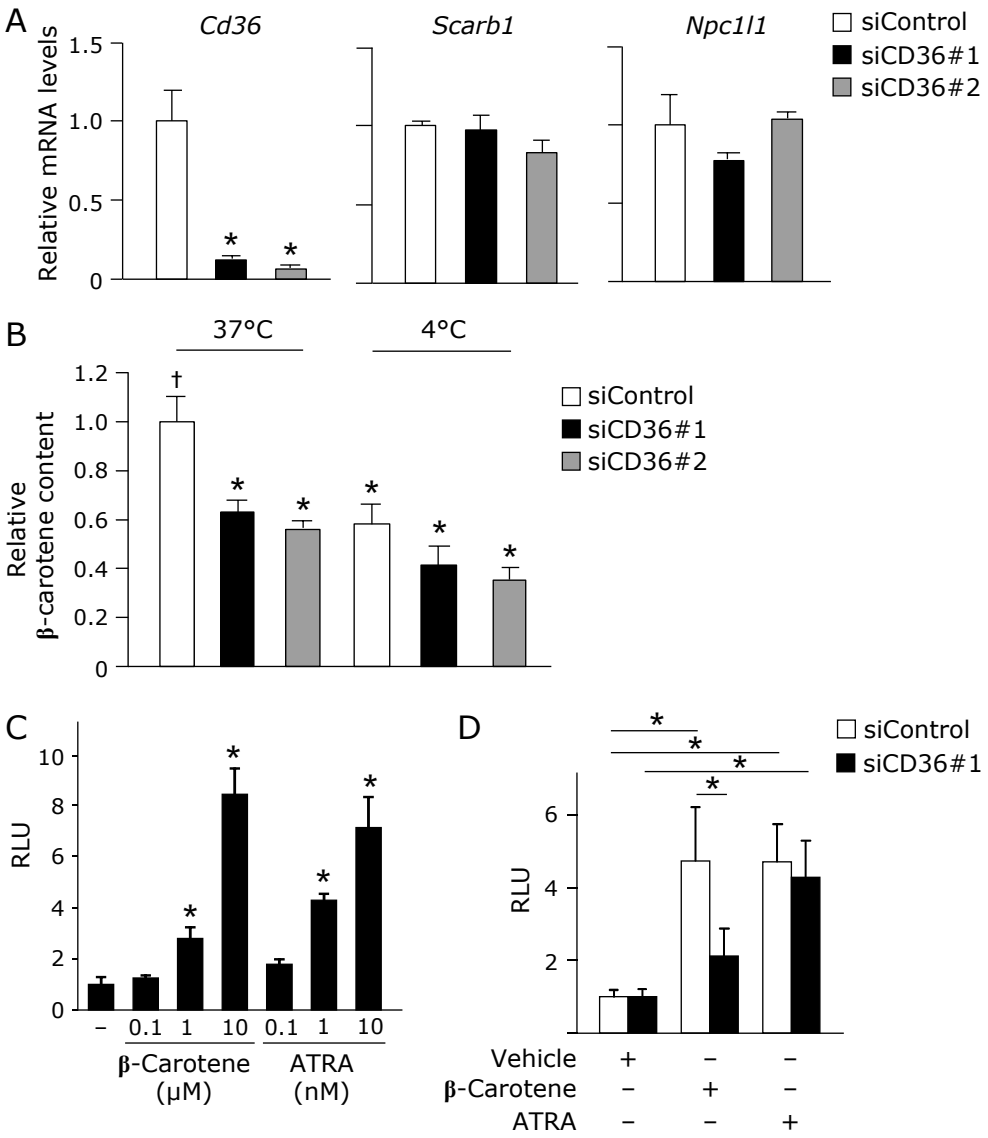

Fig. 2. Effects of *Cd36* depletion on β-carotene uptake in C2C12 myotubes. (A) C2C12 myotubes were transfected with control siRNA (siControl) or CD36 siRNA (siCD36#1 and siCD36#2), and carotenoid transporter mRNA levels were assessed by qPCR and normalized to *Actb* mRNA levels. Values are indicated as means ± SD ($n = 3$). *$p<0.05$ vs siControl, by one-way ANOVA with Dunnett's post hoc test. (B) C2C12 myotubes were transfected with control siRNA (siControl) or CD36 siRNA (siCD36#1 and siCD36#2) and then cultured in the presence of 10 μM β-carotene for 12 h at 37°C (left panel) or at 4°C (right panel). β-Carotene extracted from cells was quantified by HPLC. *$p<0.05$ vs siControl at 37°C, by two-way ANOVA with Tukey's post hoc test. †$p<0.05$ vs siControl at 4°C, by two-way ANOVA with Tukey's post hoc test. (C) C2C12 myoblasts were subjected to differentiation and were cultured in the presence of ATRA or β-carotene, and luciferase reporter activity was measured. Values are indicated as means ± SD ($n = 4$). *$p<0.05$ vs vehicle, by one-way ANOVA with Dunnett's post hoc test. (D) CD36-knocked down cells were culture in the presence of ATRA or β-carotene. Luciferase reporter activity was measured. Values are indicated as means ± SD ($n = 4$). *$p<0.05$, by two-way ANOVA with Tukey's post hoc test.

These results indicate that CD36 is involved in β-carotene-induced soleus muscle mass gain in mice and functions as the predominant transporter for β-carotene in the soleus muscles.

HIF-1α levels are higher in soleus muscles than in EDL muscles. We hypothesized that oxygen in the skeletal muscle microenvironment explains the fact that CD36 expression levels are higher in soleus muscles than in EDL muscles. To determine which of the two muscles is more hypoxic, mice were intravenously injected with the hypoxia marker pimonidazole, which binds to thiol-containing proteins in hypoxia.[21] Skeletal muscle homogenates were subjected to SDS-PAGE and protein-bound pimonidazole adducts were analyzed by Western blotting (Fig. 4A, left image). More protein-bound pimonidazole adducts were detected in soleus muscles than in EDL muscles (Fig. 4A, right panel). Furthermore, to assess the expression of HIF-1α protein in the soleus and EDL muscles, HIF-1α protein levels were normalized to β-actin protein levels. The levels of HIF1-α protein were higher in soleus muscles than in EDL muscles, even when the levels of β-actin were higher in EDL muscles than in soleus muscles (Fig. 4B). These results suggest that the intracellular environment is more hypoxic in soleus muscles than in EDL muscles.

Hypoxia induces Cd36 expression through HIF-1α in C2C12 myotubes. To determine the effects of hypoxia on the expression levels of carotenoid transporters, C2C12 myotubes were exposed to hypoxia for 12 h. Hypoxia induced *Cd36* mRNA levels, but not *Scarb1* and *Npc1l1* mRNA levels (Fig. 5A). HIF-1α protein levels were detected in myotubes under hypoxic

doi: 10.3164/jcbn.21-163

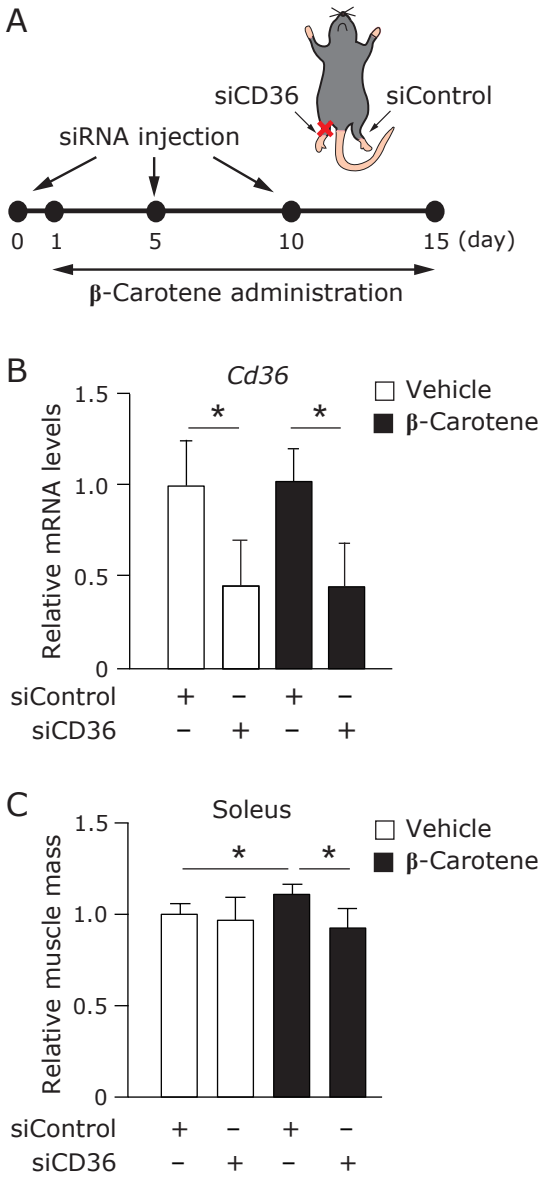

Fig. 3. Involvement of CD36 in β-carotene-induced increase in soleus muscle masses. (A) Experimental scheme. Mice in the control group and β-carotene groups were transfected with control siRNA and CD36 siRNA (siCD36#1) in the left and right soleus muscles, respectively, at days 0, 5, and 10. Mice were orally administered vehicle or β-carotene from day 1 to day 14 once daily, and the skeletal muscle was isolated at day 15. (B) *Cd36* mRNA levels were determined by qPCR and normalized to *Actb* mRNA levels. Values are indicated as means ± SD ($n = 6$). (C) The ratio of soleus muscle mass to body weight was calculated. Values are indicated as means ± SD ($n = 6$). *$p<0.05$ vs siControl/vehicle or vs siControl/β-carotene, by two-way ANOVA with Tukey's post hoc test.

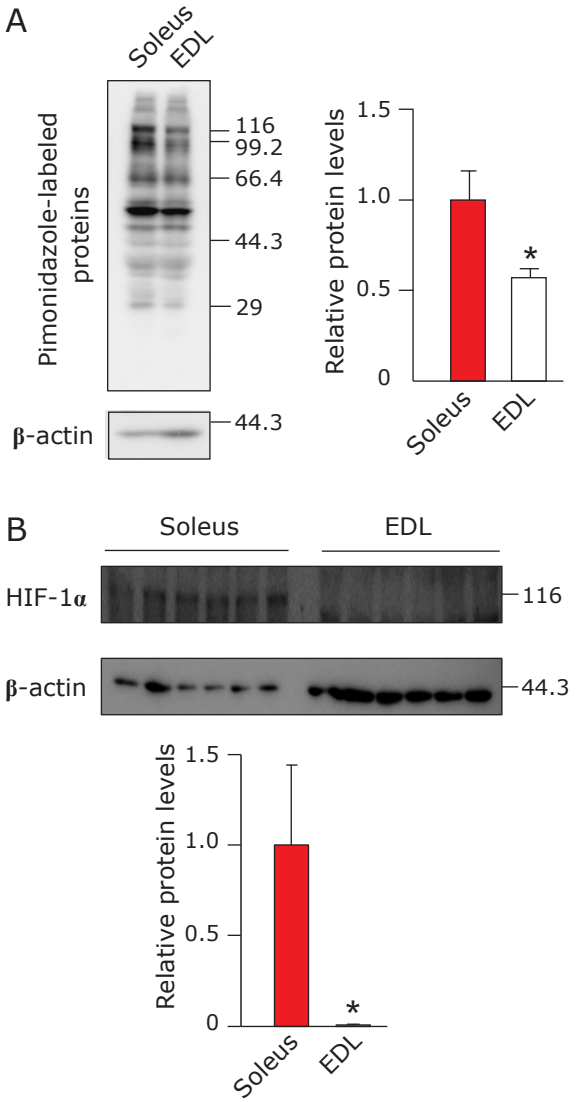

Fig. 4. Pimonidazole-conjugated proteins and HIF-1α protein in soleus muscles. (A) Mice were sacrificed 90 min after the injection of pimonidazole hydrochloride. (left panel) Tissue homogenates were analyzed by Western blotting using FITC-conjugated anti-pimonidazole antibody and peroxidase-conjugated anti-FITC antibody. (right panel) Band intensities were determined by ImageJ. Pimonidazole-conjugated protein levels were normalized to β-actin level. Values are indicated as means ± SD ($n = 6$). (B) Proteins from muscle homogenates were analyzed by Western blotting using anti-HIF-1α and β-actin antibodies. HIF-1α protein levels were normalized to β-actin levels. Values are indicated as means ± SD ($n = 6$). *$p<0.05$ vs soleus muscle, by Student's *t* test.

conditions, but not under normoxic conditions (Fig. 5B). CD36 protein levels were 1.5-fold higher in hypoxia. Furthermore, to assess whether HIF-1α is involved in the mechanism by which hypoxia induces *Cd36* expression, C2C12 myotubes were transfected with two types of HIF-1α siRNA (siHIF-1α#1 and siHIF-1α#2). Under hypoxic conditions, both HIF-1α siRNAs decreased *Hif1a* mRNA levels and significantly inhibited hypoxia-induced increase in *Cd36* mRNA levels (Fig. 5C). These results indicate that HIF-1α is involved in the hypoxic induction of *Cd36* expression in C2C12 myotubes.

HIF-1α is involved in *Cd36* expression in soleus muscles even under normal physiological conditions. To determine whether HIF-1α is involved in the regulation of CD36 expression in soleus muscles, mice were transfected with control siRNA and HIF-1α siRNA (siHIF-1α#1) in the left and right soleus muscles, respectively (Fig. 6A). Two days after the injection of siRNA, *Hif1a* mRNA level decreased to approximately 60%, and *Cd36* mRNA levels decreased to approximately 40% (Fig. 6B). These results indicate that HIF-1α is involved in *Cd36* mRNA expression in the soleus muscles even under normal physiological conditions.

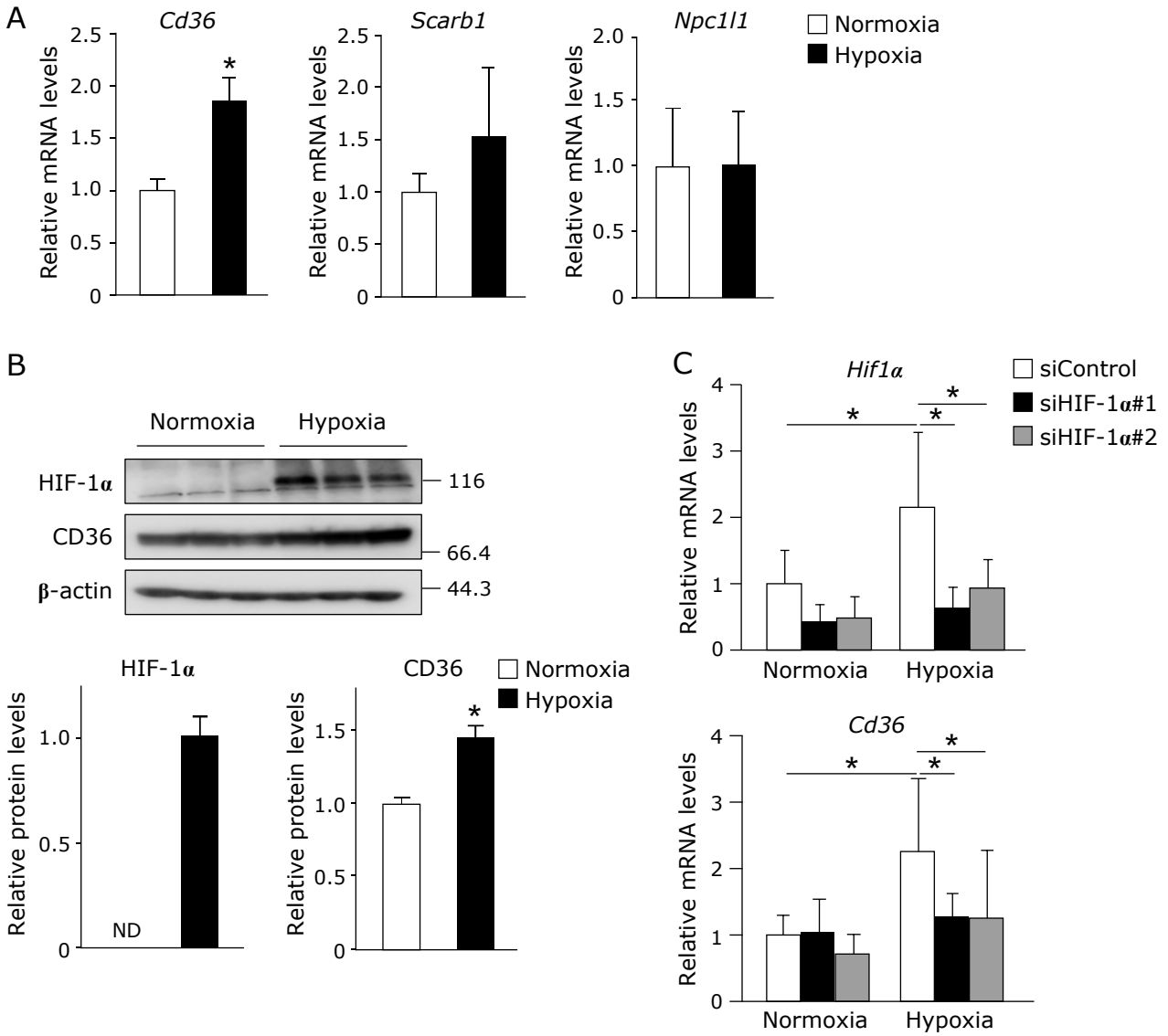

Fig. 5. Effects of hypoxia on the CD36 expression level in C2C12 myotubes. (A) Myotubes were exposed to hypoxia. The mRNA levels of carotenoid transporters, *Cd36*, *Scarb1*, and *Npc1l1*, were determined by qPCR and normalized to *Actb* mRNA levels. Values are indicated as means ± SD ($n = 3$). *$p<0.05$ vs normoxia, by Student's *t* test. (B) C2C12 myotubes were exposed to hypoxia for 12 h. (upper images) HIF-1α, CD36, and β-actin levels were analyzed by Western blotting. (lower panels) HIF-1α and CD36 levels were normalized to β-actin levels. Values are indicated as means ± SD ($n = 3$). *$p<0.05$ vs normoxia, by Student's *t* test; ND, not detected. (C) C2C12 myotubes were transfected with control siRNA (siControl) and HIF-1α siRNA (siHIF-1α#1 and siHIF-1α#2) and exposed to hypoxia for 12 h. *Hif1a* and *Cd36* mRNA levels were determined by qPCR and normalized to *Actb* mRNA levels. Values are indicated as means ± SD ($n = 4$). *$p<0.05$ vs siControl/normoxia or vs siControl/hypoxia, by two-way ANOVA with Tukey's post hoc test.

Discussion

Skeletal muscles are classified into two general types: slow-twitch muscles (e.g., soleus muscles) and fast-twitch muscles (e.g., EDL, gastrocnemius, and plantaris muscles). Our previous studies have demonstrated that dietary β-carotene induces muscle mass gain with hypertrophy in the soleus muscle, but not in the plantaris, EDL, or gastrocnemius muscles.[17] To gain information about the molecular mechanisms underlying the health benefits of β-carotene in soleus muscles, we focused on carotenoid transporters that are involved in β-carotene uptake.

The mechanisms of intestinal carotenoid absorption involve numerous complex processes, one of which is passive diffusion. The other is carrier-dependent transporter-mediated absorption.[22] In the human intestine, nearly half of dietary β-carotene is converted to all-*trans* retinal by BCO1 and the other half is absorbed intact though the rate of conversion varies widely among individuals.[23] Carotenoids are present in human blood and are incorporated into cells through carotenoid transporters.[2,3] Studies on carotenoid transporters have mainly examined carotenoid uptake into the small intestine, and at least SR-BI acts as an intestinal transporter of β-carotene.[24,25] Throughout the body, SR-BI, CD36, and NPC1L1 can all function as carotenoid transporters.[22] However, the relative contributions of these transporters to the uptake of each carotenoid in various tissues remain unclear. In the present study, the depletion of CD36 inhibited β-carotene uptake in myotubes. In addition, the depletion of CD36 inhibited RAR transcriptional activity in the presence of β-carotene, but had no influence on ATRA-activated RAR transcriptional activity. β-Carotene is converted intracellularly to all-*trans* retinal by BCO1, and all-*trans* retinal is further metabolized to ATRA. These results suggest that the depletion of CD36

doi: 10.3164/jcbn.21-163

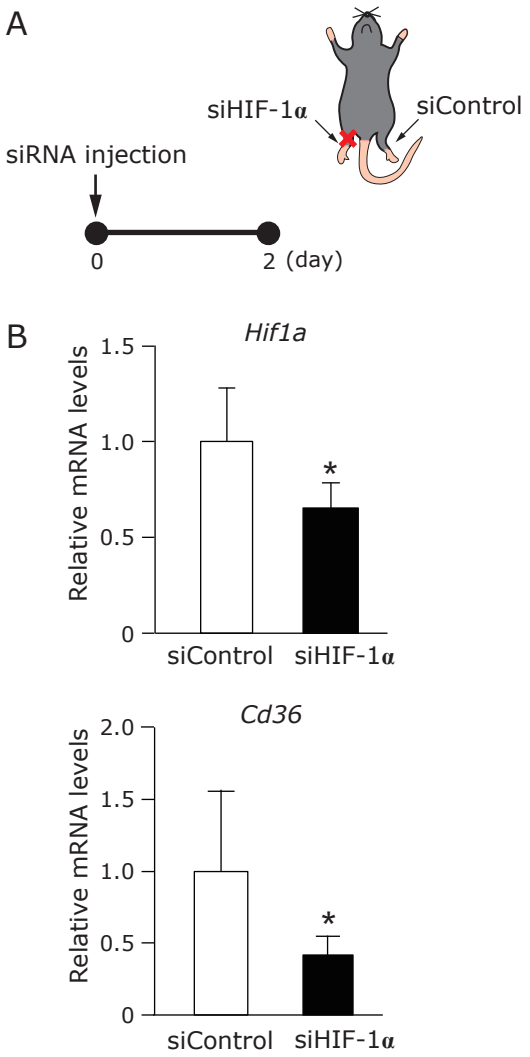

Fig. 6. Involvement of HIF-1α in *Cd36* mRNA expression levels in soleus muscles. (A) Experimental scheme. Left and right soleus muscles were injected with control siRNA and HIF-1α siRNA (siHIF-1α#1) and then isolated two days after injection. (B) *Hif1a* and *Cd36* mRNA levels were determined by qPCR and normalized to *Actb* mRNA. Data are presented as means ± SD ($n = 6$). *$p<0.05$ vs siControl, by Student's *t* test.

inhibits the uptake of β-carotene and that β-carotene is metabolized by BCO1 in myotubes. Furthermore, the knockdown of CD36 inhibited β-carotene-induced skeletal muscle mass gain. We measured the amount of β-carotene in the soleus muscle, but could not detect it. β-Carotene may have been metabolized by BCO1. Taken, together, these results indicate that CD36 is the predominant carotenoid transporter for β-carotene in soleus muscles of mice.

CD36 is expressed in broad tissues such as brown and white adipose tissue, lung, heart, mammary gland, macrophage, and skeletal muscle.[26] The expression levels of CD36 were higher in slow-twitch muscles (red muscles) than in fast-twitch muscles (white muscles). Using knockdown approach, we identified that CD36 is the main carotenoid transporter in skeletal muscle. CD36 also function as the predominant transporter that facilitates the transport of fatty acids across the plasma membrane into intestinal enterocytes, adipocytes, and skeletal myocytes.[27,28] By contrast, CD36 is required for coenzyme Q uptake into brown adipocyte, but not in soleus muscles.[29] These suggest that CD36 functions as a transporter for fatty acids and carotenoids including β-carotene, but not for CoQ10, in skeletal muscles, especially red muscle.

The pimonidazole-labeled protein levels and HIF-1α protein levels were higher in soleus muscles than in EDL muscles. The tissue oxygen level is dependent on the balance between oxygen supply and consumption.[30] The differences in capillary architecture between soleus and gastrocnemius muscles suggest that soleus muscles demand greater oxygen than gastrocnemius muscles.[9] The microvascular O_2 pressure and muscle blood flow are higher in soleus muscles than in gastrocnemius muscles, and oxygen consumption is also higher in soleus muscles than in gastrocnemius muscles.[31] Mounier *et al.*[32] report that in human muscles, HIF-1α protein levels are higher in soleus muscles (slow-twitch) than in triceps brachii muscles (fast-twitch). However, the levels of mRNA encoded by HIF-1α-responsive genes, such as vascular endothelial growth factor, are not significantly different between soleus muscles and triceps brachii muscles, and it remains unclear whether HIF-1α protein is functional in soleus muscles. Therefore, in this study, the fact that knockdown of HIF-1α reduced *Cd36* mRNA expression in soleus muscles under normal physiological conditions should provide very crucial information for elucidating the roles of HIF-1α in the slow-twitch muscles. By contrast, Pisani and Dechesne[33] report that, in murine and rat skeletal muscles, HIF-1α levels are higher in gastrocnemius, TA, and quadriceps muscles than in soleus muscles at both the protein and mRNA levels. Furthermore, in rats, HIF-1α protein levels are higher in EDL muscles than in soleus muscles, whereas *Hif-1a* mRNA levels are higher in soleus muscles than in EDL muscles.[34] The differences in HIF-1α protein levels between red slow-twitch and white fast-twitch muscles are still under debate. However, the present results from pimonidazole-bound protein levels support that the microenvironment in soleus muscles is more hypoxic than in EDL muscles.

We hypothesized that *Cd36* expression levels depend on the differences in oxygen levels in the microenvironments among different skeletal muscles. Hypoxia up-regulated CD36 expression at the protein and mRNA levels in C2C12 myotubes, and the knockdown of HIF-1α inhibited hypoxia-increased *Cd36* mRNA expression. Thus, HIF-1α functions as a positive regulator of CD36 expression in hypoxic myotubes. Hypoxia increases CD36 expression levels through HIF-1 in human monocytes and retinal pigment epithelial cells.[35,36] By contrast, hypoxia had no influence on CD36 expression level in human pancreatic β-cell line NES2Y cells and down-regulates CD36 expression levels in murine Raw264.7 macrophages and in differentiated mouse embryonic fibroblasts.[37–39] There are several alternative *Cd36* mRNA variants in mouse, rat, and human.[40–42] At least five transcript variants of murine *Cd36* mRNA are registered in GenBank, and these variants are generated by different promoter sequences. These results suggest that CD36 expression is regulated by available promoter sequences that varies from tissue to tissue. We are now attempting to determine the transcription start site of *Cd36* mRNA by 5'-RACE analysis to analyze the 5'-promoter region of *Cd36* gene of skeletal muscle.

β-Cryptoxanthin, a provitamin A carotenoid, suppresses atrophy of the soleus muscle, but not of the gastrocnemius, EDL, TA, and plantaris muscles, of senescence-accelerated mouse-prone 1 mice.[43] In contrast, dietary lycopene up-regulates MyHC I gene expression and down-regulates MyHC IIb gene expression in both soleus and gastrocnemius muscles of mice.[44] CD36 is required for lycopene uptake in adipocytes and adipose tissue.[45] The molecular mechanism by which lycopene is incorporated into the gastrocnemius muscle is unknown, but these results suggest that β-carotene, β-cryptoxanthin, and lycopene are incorporated into the soleus muscle via CD36.

In conclusion, this study demonstrates that CD36 is involved

in β-carotene-increased soleus muscle masses and that HIF-1α protein, which is stable in hypoxia, is involved in *Cd36* expression in soleus muscles. Dietary β-carotene is useful as a chemopreventive agent for the improvement of disuse-induced soleus muscle atrophy and as a naturally occurring nutraceutical for the maintenance and enhancement of soleus muscle masses in mice.[16,17] Understanding the regulatory mechanisms of *Cd36* expression presented in this study is important for enhancing β-carotene uptake in soleus muscles. On the other hand, CD36 also contributes to the uptake of fatty acids. The loss of CD36 impairs fatty acid oxidation in muscles and results in a reduced capacity for endurance running.[46] Conversely, the muscle-specific overexpression of CD36 enhances fatty acid oxidation in response to contraction and improves insulin resistance.[47,48] Further, endurance training leads to increased expression of CD36 in the soleus muscles of mice.[49,50] The hypoxic regulation of CD36 expression in soleus muscle may represent a new strategy for the maintenance and improvement of soleus muscle health involving fatty acids and carotenoids such as β-carotene.

Author Contributions

TK and RY designed the research; TK, TS, HK, AM, YK, NH, and RY analyzed data; TK, TS, HK, and AM performed the research; TK and RY wrote the manuscript; RY conceived and supervised the project.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (grant no.: 20H02937, to Ryoichi Yamaji) from the Japan Society for the Promotion of Science.

Abbreviations

ATRA	all-*trans* retinoic acid
BCO1	β-carotene 15,15'-dioxygenase 1
EDL	extensor digitorum longus
HIF-1	hypoxia inducible factor-1
MyHC	myosin heavy chain
NPC1L1	Niemann–Pick C1-like 1
qPCR	quantitative PCR
RARE	retinoic acid response elements
RARs	retinoic acid receptors
RLU	relative light units
SR-BI	scavenger receptor class B type I
TA	tibialis anterior

Conflict of Interest

No potential conflicts of interest were disclosed.

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