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Specification of skeletal muscle fiber-type is determined by the calcineurin/NFATc1 signaling pathway during muscle regeneration

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ABSTRACT

Skeletal muscle fiber type specification is changeable during muscle regeneration following cardiotoxin (CTX) injection; however, the mechanism of muscle fiber shift in regenerating muscle fibers remains unclear. Furthermore, it is unclear as to which factors determine skeletal muscle fiber types in regenerating muscle fibers. Previous studies showed that CTX-induced muscle damage resulted in a temporary hypoxic condition, indicating that hypoxia-inducible factor (HIF)-1α may be involved in muscle fiber type transition. Stabilization of HIF-1α has been shown to result in muscle fiber type transition toward slow-twitch phenotype through the calcineurin/nuclear factor activated T cell 1 (NFATc1) signaling pathway. Therefore, the aim of the present study was to determine whether the calcineurin/NFATc1 pathway is a key mediator of skeletal muscle fiber type transition during muscle regeneration. We found that CTX-induced muscle damage resulted in transient ischemia and HIF-1α expression in skeletal muscle. Additionally, it shifted the muscle fiber type proportion toward a slow-twitch phenotype in the soleus muscle (37.5% in the control muscle vs. 61.3% in the damaged muscle; $p < 0.01$) three weeks after muscle damage. Moreover, the NFATc1 protein levels increased in damaged muscle, and blockage of the calcineurin/NFATc1 signaling pathway by tacrolimus (FK-506) treatment substantially decreased the number of slow-twitch muscle fibers in the soleus muscle. This study demonstrated that CTX-induced muscle injury results in transient ischemia in hind limb muscle and stabilizes HIF-1α. Moreover, muscle damage increased oxidative phenotype muscle fibers through the calcineurin/NFATc1 signaling pathway during muscle regeneration.

1. Introduction

Skeletal muscles display different myosin heavy chain isoforms, namely type I (slow) and type IIa, b, and x (fast) [1]. Slow-twitch fibers are rich in mitochondria and myoglobin, and exhibit a predominantly oxidative metabolism, whereas fast-twitch fibers have relatively little myoglobin and capillaries, and a fast contractile speed [2]. The distribution of skeletal muscle fiber types is important in terms of metabolic regulation, as glucose uptake and insulin sensitivity are higher in oxidative fiber-dominant muscles than in glycolytic muscles [3–6]. Skeletal muscle fiber type specification is thought to be determined by genetic factors [7]; however, our previous work demonstrated that the stabilization of hypoxia-inducible factor-1α (HIF-1α) by prolyl hydroxylase domain 2 (PHD2) deficiency is closely associated with muscle fiber type transition toward oxidative fiber type through the calcineurin/NFATc1 signaling pathway [8]. Furthermore, the proportion of skeletal muscle fiber types varies during muscle regeneration after muscle damage [9]. Although the precise mechanism of muscle

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specification in regenerating muscle fibers has not been fully elucidated, there is evidence that the hypoxia-response pathway may be involved in muscle fiber type shifts during muscle fiber regeneration after damage.

Gordin et al. reported that cardiotoxin (CTX)-induced muscle damage, which is widely used to cause myonecrosis of skeletal muscle cells [10], results in a temporary hypoxic condition in the skeletal muscle [11]. A hypoxic condition is known to induce the stabilization of HIF-1α, a key transcription factor for hypoxic response including angiogenesis and glycolysis [12]. Furthermore, HIF-1α targets vascular endothelial growth factor (VEGF) and is known to be linked to angiogenesis as well as the activation of the calcineurin/nuclear factor activated T cells 1 (NFATc1) signaling pathway [13]. Activation of calcineurin results in the dephosphorylation of nuclear factor activated T-cells, the main downstream targets of calcineurin, leading to nuclear translocation and induction of slow myosin heavy chain gene expression [14].

Therefore, we focused on the possibility that the increase of slow-twitch muscle fiber type during muscle regeneration might be attributed to the activation of calcineurin/NFATc1 under HIF-1α-dependent pathway. The purpose of this study was to examine the transient hypoxic condition during muscle regeneration following CTX-induced muscle damage and consequent HIF-1α stabilization and the subsequent activation of the calcineurin/NFATc1 signaling pathway involved in muscle fiber type determination, as well as to determine the oxidative metabolic capacity according to skeletal muscle fiber shift.

2. Materials and methods

Ethical approval

All animal experimental procedures were performed according to protocols approved by the Guidelines for the Care of Laboratory Animals of Tohoku University Graduate School of Medicine (Sendai, Japan).

2.1. Animals

The 10–12 weeks old male wild-type C57/Bl6 mice were anesthetized and subsequently injected with 100 μl of either CTX from *Naja mossambica mossambica* (Sigma-Aldrich, C9759, St.Louis MO, USA) dissolved at 10 μM in saline percutaneously into the calf of right limb to induce muscle damage in gastrocnemius, plantaris and soleus muscles, and 100 μl of saline into the calf of left limb. The mice were sacrificed at 3, 7, 14, 21, 28, and 84 days after injury. FK-506 (ALX-380-008, Enzo Life Sciences, Farmingdale, NY, USA) was dissolved in ethanol and phosphate buffer solution (PBS) and administered via intraperitoneal (i.p.) injection at a dose of 1 mg kg/d after muscle injury.

2.2. Western blot

To isolate total protein extracts, 50 mg of skeletal muscle tissue was homogenized for 30 s on ice in 1 mL of lysate buffer (40 mM Tris (pH 7.5), 300 mM KCl, 1% Triton X-100, 0.5 M EDTA) using a Polytron PT-MR 2100 homogenizer. Homogenates were centrifuged at 12,000 rpm for 5 min at 4 °C, and the supernatants were isolated. The nuclear and cytoplasm were extracted by NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher, #78833, Rockford, IL, USA). Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) with bovine serum albumin (BSA) as the standard, and extracts were stored at −80 °C. Total protein was separated by 8–12% SDS-PAGE and transferred to a PDVF membrane (Invitrogen). The membrane was blocked using Tris-buffered saline with 0.05% Tween 20 (TBST) containing 5% BSA for 1 h and incubated overnight with appropriately diluted (1:500–1000) primary antibody in TBST at 4 °C. After incubation, the membranes were rinsed thrice in TBST for 5 min and incubated with secondary antibodies in 4% skim milk for 1 h at room temperature. Protein bands were visualized and quantified using a Molecular Imager VersaDoc 5000 MP system (Bio-Rad) and ECL GE Healthcare Amersham ECL Prime western blotting detection reagent (GE Health care, Buckinghamshire, UK.

2.3. Antibodies

The following primary antibodies were used: Monoclonal Anti-Myosin slow (M8421, Sigma, St Louis, MO, USA), Anti-Calcineurin pan A (07–1491, Sigma, St Louis, MO, USA), HIF-1α (NB100-479, Novus, Centennial, CO, USA), NFATc1 (sc-7294, Santa Cruz Biotechnology, Oregon, USA), VEGF (sc-7269, Santa Cruz Biotechnology, Oregon, USA), GAPDH (14C10, Cell signaling Technology, Danvers, MA, USA).

2.4. Determination of muscle fiber-type composition

Gastrocnemius and soleus muscle samples were harvested from mice (five mice per each group), snap frozen, and embedded in Tissue-Tek O·C.T. compound (Sakura, Torrance, CA, USA) in methylbutane for cryosectioning. The skeletal muscle cryosections were air-dried for 10 min and fixed in 4% paraformaldehyde for 15 min. For muscle fiber type composition experiments, the sections were blocked in PBS containing 0.3% Triton X-100 and M.O.M mouse blocking reagent (Vector Laboratories, MKB-2213, Newark, USA) for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C. We used several primary antibodies, including Monoclonal Anti-Myosin slow (M8421, Sigma, St. Louis, MO. USA), myosin heavy chain 2A (SC-71, Developmental Studies Hybridoma Bank, Iowa), anti-laminin antibody (L9393, Sigma-Aldrich St. Louis, MO. USA) in the blocking solution with M.O.M protein concentrate (Vector Laboratories, MKB-2213, Newark, USA). The sections were then incubated with secondary antibodies in PBS for 1 h at room temperature. We used several secondary antibodies, including Alexa Fluor 488 Goat Anti-mouse IgG and Alexa Fluor 555 Goat Anti-rabbit IgG in PBS containing 0.3% Triton X-100. All slides were covered with VECTASHIELD mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). The samples were visualized under a microscope (C2+, Nikon, Tokyo, Japan) and analyzed using NIS Elements and ImageJ software version 1.48u4 (National Institutes of Health, Bethesda, MD, USA). The proportion of muscle fiber types in whole sections of the soleus and gastrocnemius muscles was analyzed.

2.5. Determination of capillary density

Frozen sections were blocked in PBS containing 0.3% Triton X-100 and 10% goat serum (Sigma-Aldrich, St louis, MO, USA) for 1 h at room temperature. Anti-mouse CD31 (PECAM-1, BD Pharmingen, San Diego, CA, USA) was used as the primary antibody and was incubated overnight in blocking solution at 4 °C. Alexa Fluor 488 Goat Anti-Rat IgG (Life Technology, Carlsbad, CA, USA) in blocking solution was used as the secondary antibody for 1 h at room temperature. All slides were covered with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA). Detection was performed using a microscope (C2+, Nikon, Tokyo, Japan), and the images were analyzed using NIS Elements software version 1.48u4 (National Institutes of Health, Bethesda, MD, USA). Fields of whole muscle tissue cross-sections from the soleus and gastrocnemius were selected for CD31-positive cell counting and analyzed using

ImageJ software.

2.6. Laser Doppler perfusion imaging

Briefly, excess hair was removed from the hind limbs of the mice. Blood perfusion in mouse hind limbs was assessed using laser doppler imaging system (MoorLDI2-IR; Moor Instruments, Delaware, DE, USA). For each mouse, blood perfusion was measured in both control and damaged legs, and the ratio was calculated with original software (RESEARCH Version 3.09; Moor Instruments, Delaware, DE, USA). The results were expressed as the mean ratio of damage to control values for each experimental group.

2.7. Succinate dehydrogenase staining

Sections of the gastrocnemius muscles were stained with succinate dehydrogenase (SDH). The sections were first allowed to dry at room temperature and were then incubated in a solution containing nitro blue tetrazolium (0.5 mg mL^{-1}), sodium succinate (50 mM), and phosphate buffer (50 mM) for 50 min at 37 °C. The cross-sections were then washed three times with distilled water and cover-slipped using a glycerol-based mounting medium [15].

2.8. RNA extraction and real-time quantitative PCR

Total RNA was isolated from muscle tissues using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Copy DNA (cDNA) templates were obtained via reverse transcription of 2 μg of total RNA (QuantiTect Reverse Transcription; Qiagen). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in a 96-well plate using the Fast SYBR Green PCR master mix (Life Technologies Cor., Carlsbad, CA, USA) and the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows: 95 °C for 7 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, and a final extension of 95 °C for 15 s and 60 °C for 30 s. The relative expression was calculated using the standard method. All values for each gene were averaged and normalized to β-actin or 18S rRNA as internal controls.

2.9. Statistics

All results are expressed as the means, with error bars representing the standard error or standard deviation. Two-tailed Student's t-tests were performed to determine p values. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test or Fisher's LSD test was used to compare the different groups. All statistical analyses were performed using Graph pad Prism 9.4.0 software.

3. Results

3.1. Alteration of skeletal muscle phenotype after CTX-induced muscle damage

To investigate the change in the muscle fiber type proportion during muscle regeneration, we examined histological sections from the soleus, plantaris, and gastrocnemius muscles on days 3, 7, 14, and 21 weeks after CTX injection (Fig. 1A). We analyzed gastrocnemius and plantaris together because CTX injection may result in the adhesion of three calf muscles, and it was especially difficult to isolate gastrocnemius and plantaris. Although the damaged gastrocnemius and plantaris muscles showed a significant decrease in muscle weight on days 3 and 7 after CTX injection, both recovered at day 21. In contrast, CTX injection did not alter soleus muscle weight (Fig. 1 B and C). We also found that muscle damage caused by CTX resulted in a muscle fiber type transition toward a slow-twitch phenotype in both the soleus and gastrocnemius and plantaris muscles. The proportion of slow muscle fibers increased significantly on day 21 in the gastrocnemius and plantaris muscles (Fig. 1D–E). Furthermore, the proportion of slow-twitch muscle fibers in the damaged muscle increased in the soleus (37.5% in the control muscle vs. 61.3% in the damaged muscle, $p < 0.01$) at 21 days after CTX injection. In contrast, the proportion of type IIa fibers significantly decreased in the soleus muscle (47.3% in the control muscle vs. 33.5% in the damaged muscle, $p < 0.01$) after CTX injection. Additionally, the proportion of type IIx and b fibers decreased significantly after CTX injection in both the soleus (15.1% in control muscle vs. 5.2% in damaged muscle, $p < 0.01$) and gastrocnemius (91.8% in control muscle vs. 86.8% in damaged muscle, $p < 0.01$) (Fig. 1F–G). Thus, the muscle damage induced by CTX increases the number of slow-twitch muscle fibers during regeneration.

3.2. Transient ischemia by muscle damage may alter muscle fiber proportion via hypoxic response pathway

To assess whether blood vessels injured by CTX injection impair blood flow in skeletal muscles, we monitored the skeletal muscle blood flow in CTX-injected muscles using a laser doppler. We observed impairment of blood flow on day 7 after muscle damage (Fig. 2A) in the damaged muscle compared with the control. Additionally, the damaged muscle had a lower angiographic score (ischemic/non-ischemic ratio) on day 7 than before injury (Fig. 2B).

To explore whether the impaired blood flow in damaged muscle was linked to hypoxic condition in skeletal muscle tissue, we analyzed HIF-1α, a key mediator of the hypoxic response pathway, and VEGF in the gastrocnemius. We found that CTX-induced muscle damage resulted in increased HIF-1α and VEGF expression at day 3 after muscle damage in the gastrocnemius (Fig. 3A–C). Furthermore, we observed an increase in nuclear NFATc1 expression in the damaged gastrocnemius muscle compared to that in the control muscle on day 3 (Fig. 3E and F).

To investigate the role of the calcineurin/NFATc1 signaling pathway in slow-twitch muscle fiber formation during muscle regeneration, we administered FK-506, a calcineurin inhibitor, for 14 days starting from day 7 after muscle damage, blunting the increase in slow-twitch muscle fiber formation in the soleus muscle during muscle regeneration. Interestingly, FK-506 treatment for 2 weeks starting 1 week after muscle damage resulted in a significant decrease in type I muscle fibers and a significant increase in type IIx and b fibers at 3 weeks after muscle damage in the soleus muscle (Fig. 4A–C). This indicates that the specification of muscle fiber type is determined by the calcineurin/NFATc1 signaling pathway following muscle damage.

3.3. Increase in oxidative muscle fiber type following muscle damage is not linked to alteration of skeletal muscle metabolism and capillary formation

To evaluate skeletal muscle angiogenesis during muscle regeneration, we analyzed the expression of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor 2 (VEGFR2) in gastrocnemius muscle. VEGFR2 expression was increased in the damaged gastrocnemius muscle compared to the uninjured muscle on days 3 and 7 (Fig. 5A–D). Tubulin content is known to be more abundant in MHC-I/a dominant muscles than in MHC-II dominant muscles [16]. In line with this, we found that β-tubulin expression levels were higher in the soleus muscle containing higher MHC-1/a compared to gastrocnemius and TA

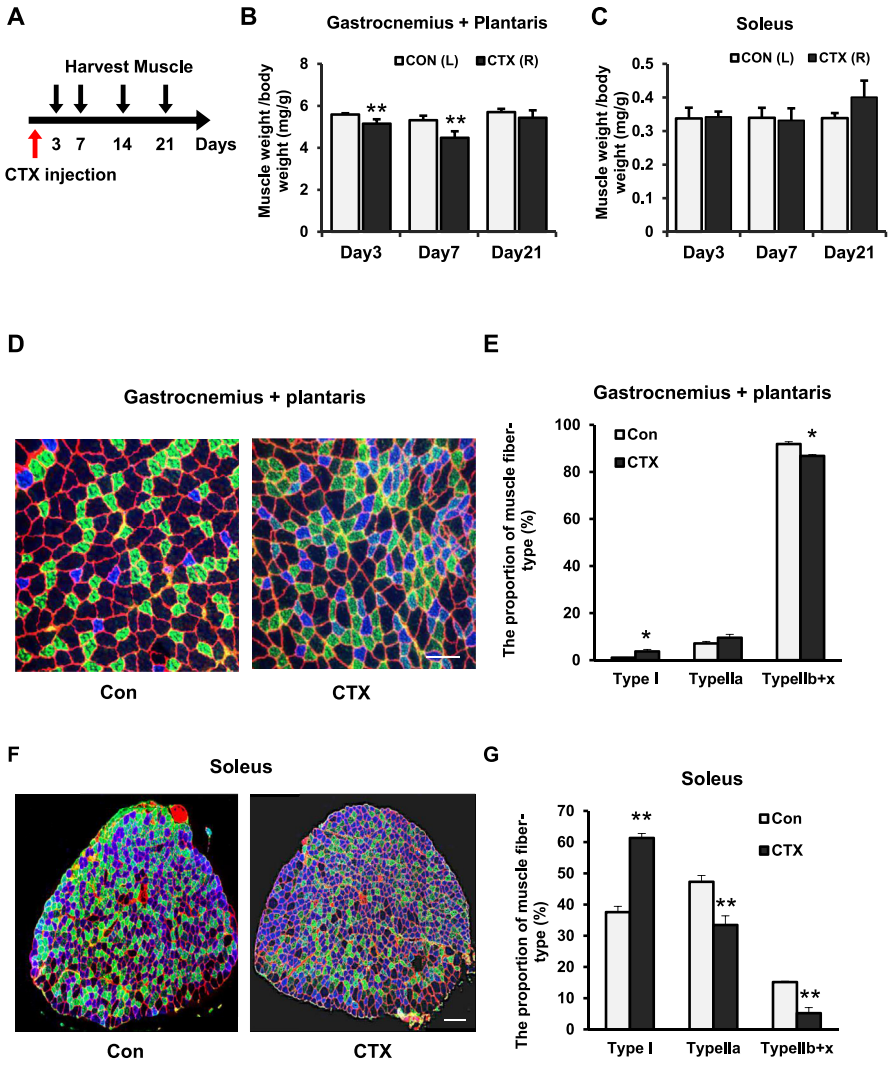

Fig. 1. Muscle damage induced by cardiotoxin (CTX) induced muscle fiber type shift toward slow-twitch phenotype in skeletal muscle. A. Experimental design. B and C. Alteration of muscle weight of both gastrocnemius and plantaris, and soleus after CTX injection. D. Representative images of muscle fiber type in gastrocnemius and plantaris muscle (Blue: type I, Green: type IIa, and unstained: type IIb and x). E. Quantification of composition of muscle fiber type at 21 days after CTX injection in gastrocnemius and plantaris muscle. F. Representative images of muscle fiber type in soleus muscle (Blue: type I, Green: type IIa, and unstained: type IIb and x). G. Quantification of the proportion of muscle fiber type in control and damaged soleus muscle at 21 days after CTX injection. N = 3–5 per group, *p < 0.05, **p < 0.01 compared to control. Values are means ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

muscles. Additionally, the soleus muscle expressed higher levels of VEGFR2 compared with the gastrocnemius and TA muscles (Fig. 5E). Indeed, we observed higher expression of both β-tubulin and VEGFR2 in CTX-injected muscles. This may reflect that muscle fibers are formed as slow-twitch fibers during regeneration.

Furthermore, muscle damage caused by CTX injection showed a significant increase of CD31-positive cells in the soleus muscle on day 21. To analyze the relationship between slow-twitch muscle fiber formation and capillary formation, we assessed capillary density in FK-506-treated soleus muscle. FK-506 treatment for 2 weeks resulted in a substantial decrease in slow-twitch muscle fibers; however, the number of CD31 positive cells increased due to muscle damage (Fig. 6A and B).

To assess the alteration of enzymes associated with skeletal muscle metabolism depending on muscle fiber type, we analyzed the alteration in muscle fiber type, expression of several genes related to oxidative metabolism, and SDH staining at the termination point of muscle regeneration (12 weeks after muscle damage). We observed increased slow-twitch muscle fibers due to muscle damage at 12 weeks after muscle damage (Fig. 6A and B); however, there were no significant differences in the expression of genes implicated in oxidative metabolism, such as ATG5g1 and COX5 (Fig. 6C) Additionally, SDH enzyme activity was not significantly altered by the muscle fiber type transition toward the oxidative phenotype at 12 weeks after muscle damage (Fig. 6D).

4. Discussion

Skeletal muscle repair is a highly complex process involving the coordinated phases of degeneration, inflammation, regeneration, and fibrosis [17]. Muscle damage activates quiescent satellite cells residing in the basal lamina, which proliferate and differentiate into myoblasts during regeneration [17,18]. Many studies have revealed the mechanism and role of satellite cells during muscle regeneration after damage. However, there is little information on the mechanism by which the specification of muscle fiber type is determined in the regenerating muscle. Matsuura et al. demonstrated that muscle damage induced by CTX causes an increase in

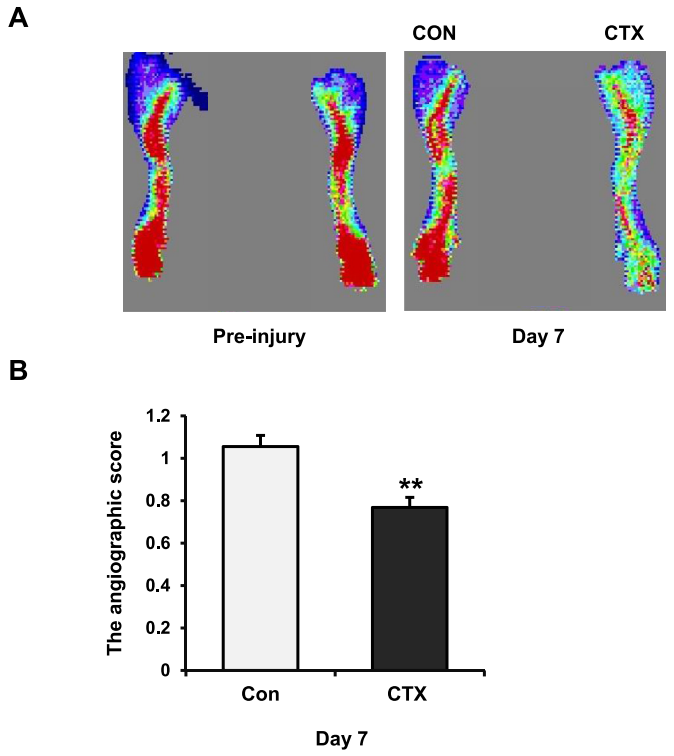

Fig. 2. Cardiotoxin (CTX) injection induced decrease of blood flow in hind limb muscle at 7 days after muscle damage. A. Blood flow in hind limb muscle was measured via laser doppler before injury and at 7 days after CTX injection. B. Evaluation of the angiogenic score (n = 4–6 per group). *p < 0.05, compared to control. Values are means ± SEM.

slow-twitch muscle fibers 28 days after CTX injection [9]. We also found an increase in the proportion of slow-twitch fibers in both the soleus and gastrocnemius muscles at 3 weeks after CTX-induced muscle damage. This indicates that the specification of the muscle fiber type may be determined before 3 weeks after muscle damage. Gondin et al. reported that CTX-induced muscle damage showed temporary hypoxic condition at days 2, 4, and 8 as well as an increase in HIF-1α expression after muscle damage [11]. Here, we observed restricted blood flow in hind-limb using laser doppler and the stabilization of HIF-1α in skeletal muscle at day 3 after CTX injection. HIF-1α stabilization is known to trigger the expression of genes associated with angiogenesis, erythropoiesis, and energy metabolism [19]. Moreover, we observed an increase in VEGF, a target of HIF-1α, as well as a significant increase in capillary density in the damaged muscle. The transient hypoxic condition might be a crucial for inducing the stabilization of HIF-1α and angiogenesis in skeletal muscle.

Furthermore, HIF-1α stabilization is involved in muscle fiber type transition toward the oxidative phenotype. Our previous study showed that the stabilization of HIF-1α by PHD2 deficiency and 10% of actual hypoxic condition induced a muscle fiber type transition toward oxidative twitch muscle fiber via calcineurin/NFATc1 signaling pathway in mice [20]. Thus, we focused on the possibility that the hypoxia-response pathway under transient ischemia after muscle damage results in the formation of slow-phenotype fibers through the calcineurin/NFATc1 signaling pathway.

4.1. Specification of muscle fiber type via calcineurin/NFATc1 signaling pathway after muscle damage

The serine/threonine protein phosphatase calcineurin is

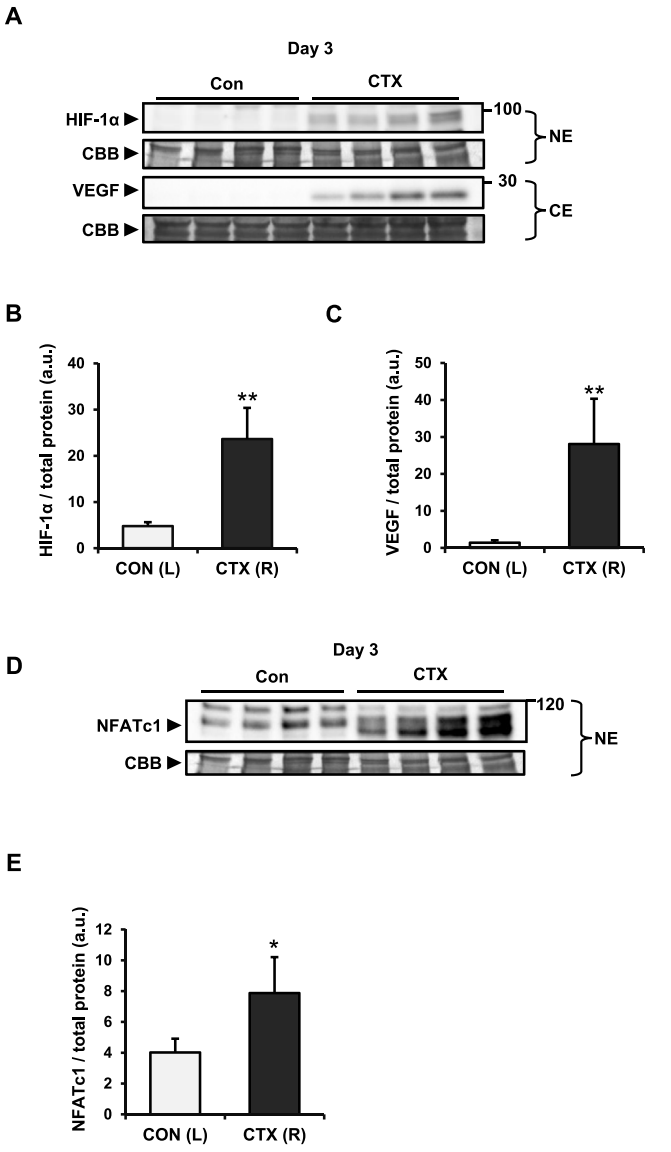

Fig. 3. Muscle damage induced the expression of hypoxia-inducible factor-1α (HIF-1α) and nuclear translocation of NFATc1. A. Protein expression of HIF-1α and VEGF were measured by western blotting in gastrocnemius 3 days after cardiotoxin (CTX) injection. B and C. Quantification of HIF-1α and VEGF expression in control and injured gastrocnemius muscle. D. Nuclear NFATc1 in gastrocnemius at day 3 after CTX injection. E. Quantification of nuclear NFATc1 in control and injured gastrocnemius muscle (n = 4). *p < 0.05, **p < 0.01 compared to control. Values are means ± SD.

regulated by intracellular calcium concentration and plays an essential role in NFATc1 translocation into the nucleus. NFATc1 transcription complexes modulate numerous genes necessary for cell-cell interactions related to immune response signals [21]. Interestingly, NFATc1 directly expresses slow-twitch myosin light chains in muscle cells [22]. Furthermore, recent studies showed that TRPM2/ca2+/NFATc1 signaling plays a pivotal role in exercise-induced muscle fiber type transitions to slow-twitch muscle [23]. Thus, the translocation of NFATc1 into the nucleus by calcineurin activation is strongly linked to the muscle fiber shift toward slow twitch [24]. Here, we observed nuclear translocation of NFATc1 in the gastrocnemius muscle on day 7 after CTX injection and an increase in slow-twitch muscle fibers in the regenerating muscle. This indicates that the NFATc1/calcineurin axis may be a key modulator of the specification of new muscle fibers during muscle

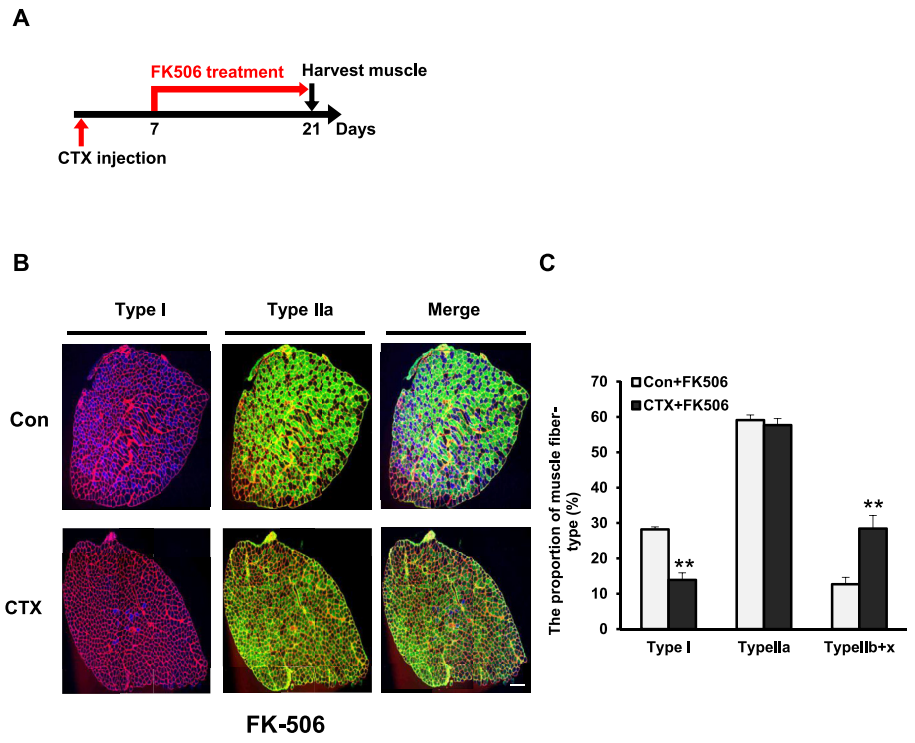

Fig. 4. Administration of FK-506, a calcineurin inhibitor, resulted in a decrease of slow-twitch fibers in soleus muscle at 3 weeks after muscle damage. A. Experimental schematic outlining the cardiotoxin (CTX) injection and FK-506 treatment. B. Representative images of muscle fiber type in soleus (Blue: type I, Green: type IIa, and unstained: type IIb and x). C. Quantification of proportion of muscle fiber type in soleus muscle with FK-506 treatment for 7 days. ($n = 5$ per group). *$p < 0.05$ **$p < 0.01$ compared to control. Values are means ± SEM. (scale bar = 100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

regeneration. To investigate whether calcineurin modulates muscle fiber specification during muscle regeneration, we inhibited calcineurin activity by administering FK-506, a calcineurin inhibitor. We started FK-506 administration on day 1 after CTX injection for 7 days to verify whether the calcineurin pathway is involved in slow-twitch muscle fiber formation in the muscle cell proliferation and differentiation process as muscle damage causes an increase in the expression of genes, such as Pax7, MyoD, and Myogenin, upon satellite cell proliferation and differentiation within 7 days of damage [25]. The inhibition of calcineurin by FK-506 treatment from day 1 for 7 days did not suppress slow-twitch muscle fiber formation in the soleus muscle. Subsequently, we administered FK-506 on days 7–14 because myofiber regeneration is initiated on day 7 after damage [25]. Interestingly, we found that FK-506 treatment starting on day 7 considerably suppressed slow-twitch fiber formation and increased the number of fast-twitch fibers. These results indicate that the calcineurin/NFATc1 axis is essential for slow-twitch muscle fiber formation between days 7 and 14 of muscle regeneration.

In contrast, the calcineurin pathway does not play a role in neovascularization during skeletal muscle regeneration. Although inactivation of the calcineurin/NFATc1 pathway inhibited slow-twitch muscle fiber formation after muscle damage, capillary density increased significantly in the soleus muscle, regardless of the muscle fiber type transition. These results suggest that the calcineurin/NFATc1 signaling pathway is not involved in angiogenesis during skeletal muscle regeneration. This is consistent with a previous report that capillary formation is not required for muscle fiber type transition toward the oxidative phenotype [26]. Thus, skeletal muscle angiogenesis is not likely to be associated with slow-twitch fiber formation; however, the expression of VEGF in skeletal muscle by HIF-1α may be involved in muscle fiber type transition, because VEGF is known to regulate the calcineurin/NFATc1 signaling pathway in endothelial cells [27]. Additionally, we evaluated the alteration in skeletal muscle metabolism depending on the increase in slow-twitch muscle fiber type proportion because slow-twitch fibers rely more heavily on oxidative metabolism than fast-twitch fibers. To evaluate the alteration of skeletal muscle metabolism, we measured SDH activity using SDH staining and the mRNA level of gene expression related to oxidative metabolism, such as that of ATP synthase H+ transporting mitochondrial F1 complex (ATP5g1), and cytochrome *c* oxidase subunit V a (Cox5a). However, we did not observe any notable alteration of oxidative metabolism in gastrocnemius at 12 weeks after muscle damage in spite of the enriched slow-twitch muscle fibers. This indicates that the change in oxidative muscle fiber formation in the calcineurin/NFATc1 signaling pathway does not assure functional alterations, such as skeletal muscle energy metabolism. Based on our previous studies on the effects of exercise training, exercise training is necessary to activate oxidative metabolism rather than the muscle fiber transition [15].

This study demonstrated that CTX-induced muscle injury caused transient ischemia in hind limb muscle and HIF-1α stabilization. The transient ischemic condition caused by muscle damage enhances the increase in the oxidative phenotype of muscle fibers through the calcineurin/NFATc1 signaling pathway during muscle regeneration. Although our study could not be applied to a traumatic muscle injury model or to investigate the effect of inflammation on muscle damage, we revealed that the activation of the calcineurin/NFATc1 signaling pathway by muscle damage is strongly involved in the muscle fiber type transition toward a slow-twitch phenotype during muscle regeneration. Moreover, males and females have different muscle fiber-type compositions and adaptive responses to hypoxia [28–30]. Thus, future studies are

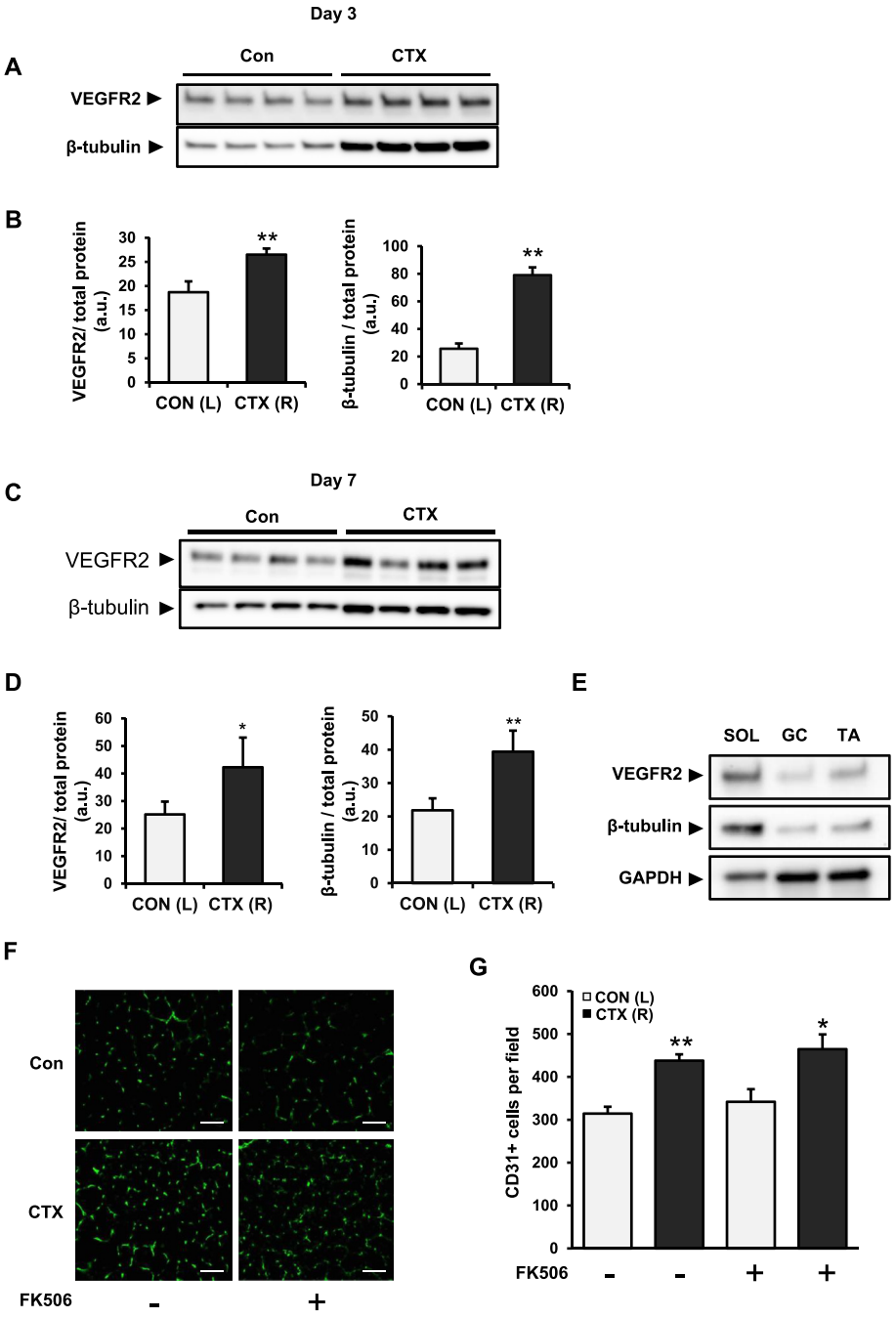

Fig. 5. Expression of vascular endothelial growth factor receptor (VEGFR) in gastrocnemius muscle and capillary density in soleus muscle after cardiotoxin (CTX) injection. A and B. VEGFR expression in gastrocnemius muscle at day 3 after CTX injection. C and D. VEGFR expression in gastrocnemius muscle at 7 days after CTX injection. E. VEGFR2 expression in skeletal muscle (soleus, gastrocnemius, and tibialis anterior) ($n = 4$ per group). F and G. Capillary density in skeletal muscles was determined by detecting CD31-positive cells using immunostaining ($n = 3$–4 per group). *$p < 0.05$ **$p < 0.01$ compared to control. Values are means ± SD (scale bar = 100 μm).

needed to examine the biological gender difference in muscle fiber-type transition. Our findings provide new insights for understanding muscle fiber specification during muscle regeneration.

Author contributions

JS, AN, and RN contributed substantially to the conception and design. JS and AN performed data acquisition and analysis and interpreted experimental results. JS and RN drafted the manuscript. RN and KG edited and revised the manuscript. All authors approved the final version of the manuscript for publication and agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

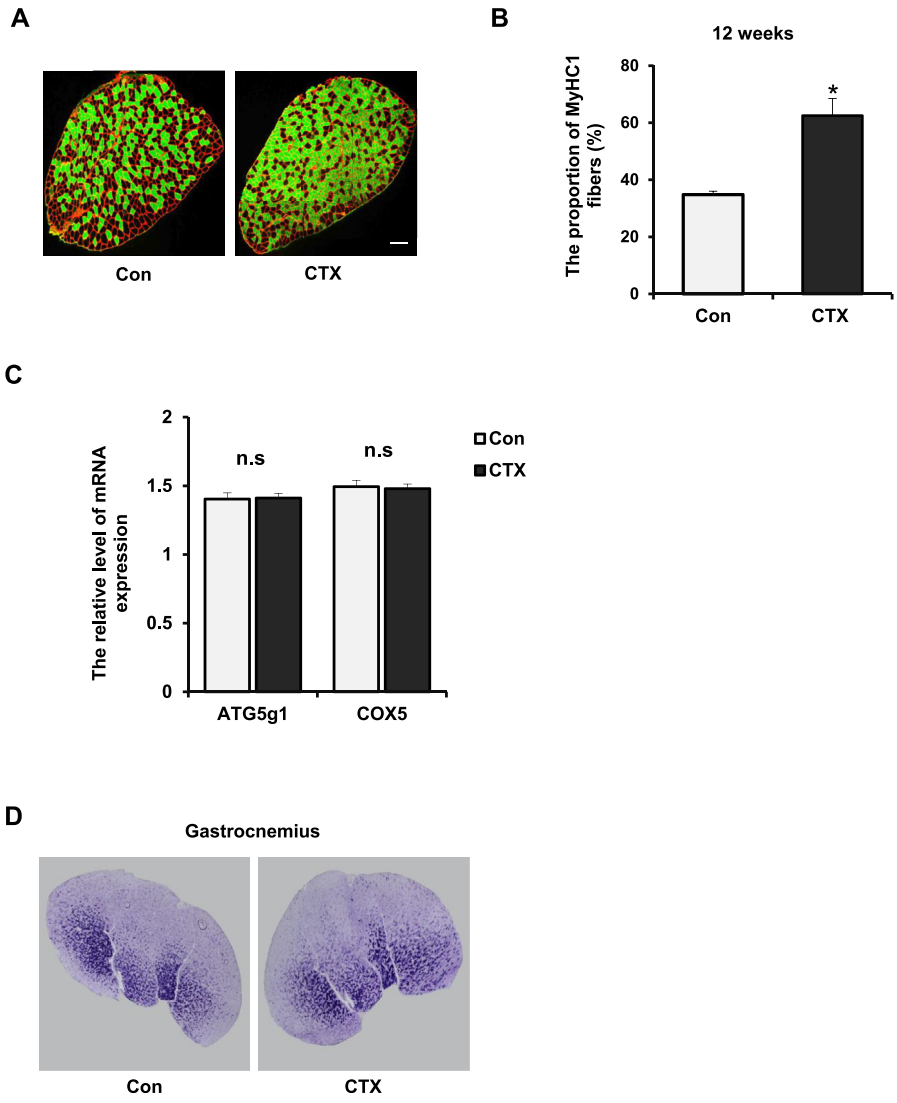

Fig. 6. Alteration of muscle fiber phenotype toward slow-twitch **fibers was not accompanied by activation of oxidative metabolism.** A. Frozen sections of soleus muscle at 12 weeks after muscle damage were stained with antibodies for type I/slow (blue) and type IIa (green) fibers and laminin (red). B. mRNA levels of genes involved in oxidative metabolism, such as ATG5g1, and COX5 ($n = 3$ per group). C. Succinyl dehydrogenase (SDH) staining of the gastrocnemius and plantaris muscle. *p < 0.05, **p < 0.01 compared to control. Values are means ± SEM. (scale bar = 100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.03.032.

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