Differential Effects of Astaxanthin on Oxidative Key Enzyme and Capillarization in the Deep and Superficial Layers of Unloading-induced Atrophied Muscle

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Abstract. [Purpose] The fiber type composition of the muscle hypothetically causes differences in the effects of astaxanthin on capillary regression in the superficial and deep layers of atrophied muscle. The effect of astaxanthin on capillary regression was studied in the superficial and deep layers of the plantaris muscle. [Subjects and Methods] Twenty-four Wistar rats were divided into 4 groups: control, control treated with astaxanthin, hind limb unloading, and hind limb unloading treated with astaxanthin groups. The cross-sectional area and succinate dehydrogenase activity of the plantaris muscle fibers, capillary-to-muscle fiber ratio, and number of capillaries around the muscle fiber were measured using histological images. [Results] In the deep layer, succinate dehydrogenase was activated, and the number of capillaries was decreased in atrophied plantaris muscle. However, these changes were not found in the superficial layer. [Conclusion] Capillary regression in atrophied skeletal muscle was initiated in the deep layer, where muscle fiber characterized by high mitochondrial oxidative enzyme activity is predominantly located, and astaxanthin administration effectively prevent capillary regression.

Key words: Astaxanthin, Capillary, Atrophied muscle

(INTRODUCTION

Skeletal muscle fibers are categorized as oxidative and glycolic fibers on the basis of their metabolic properties. Compared to muscle with more glycolic fibers, muscle with more oxidative fibers has higher oxidative enzyme activity and capillary networks. Muscle atrophy results from inactivity, through unloading or immobilization, and oxidative fibers are more vulnerable to muscle atrophy than glycolic fibers. Muscle atrophy involves capillary regression, which is also more marked in oxidative muscle than in glycolic muscle. It has been reported that inactivity results in overexpression of reactive oxygen species in atrophied muscle, leading to capillary regression.

Astaxanthin is a red carotenoid pigment and an antioxidant. In a pilot study, we investigated the effect of astaxanthin on capillary regression in the rat soleus muscle, and found that capillary regression in the atrophied soleus muscle, induced by unloading, was prevented by astaxanthin administration (data not shown). Capillary regression in muscle atrophy is related to oxidative stress, such as overexpression of reactive oxygen species. As an antioxidant, astaxanthin was effective at attenuating oxidative stress and inhibiting capillary regression.

The rat soleus muscle, the focus of our pilot study, mainly consists of oxidative fibers. However, most skeletal muscles are comprised of both oxidative and glycolic fibers. In addition, the fiber type composition of the muscle is different in the superficial and deep layers in most skeletal muscles. For example, the plantaris muscle has more oxidative fibers located in the deep layer than in the superficial layer. Therefore, we hypothesized that the fiber composition of the muscle would lead to different effects of astaxanthin on capillary regression in the superficial and deep layers of atrophied muscle.

Whether the preventive effect of astaxanthin on capillary regression depends on the composition of muscle fiber types has not yet been determined. Knowledge of the effect of astaxanthin on different muscle fiber types would improve the management of dysfunction in atrophied muscle, such as circulation and oxygen insufficiencies.

Therefore, we examined the effect of astaxanthin on capillary regression in the superficial and deep layers of the atrophied plantaris muscle. The purpose of the present study was to elucidate the differences between the superficial and deep layers with regard to the efficacy of astaxanthin on capillary regression in atrophied muscle.)
SUBJECTS AND METHODS

This study was approved by the Institution Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations. Twenty-four 12-week-old male Wistar rats (Japan SLC, Japan) were divided into 4 groups: control (Cont), control treated with astaxanthin (CA), hind limb unloading (HU), and hind limb unloading treated with astaxanthin (HA) groups. Astaxanthin (Fuji Chemical Industry, Japan) was orally administered at 50 mg/kg twice a day for 1 week. For hind limb unloading, animals were suspended by a string attached to their tails to prevent weight bearing on the hind limbs for a week\textsuperscript{11}. Each animal was individually housed in an isolated room which was environmentally controlled at 25 °C under a 12:12-h light-dark cycle.

The plantaris muscles were surgically removed under anesthesia with intra-peritoneal administration of pentobarbital sodium (50 mg/kg) and their wet weights were measured. The muscle samples were immediately frozen in acetone, cooled using dry ice, and stored at −80 °C until the analyses. The muscle samples were cut on a cryostat (CM1510s, Leica, Germany) at −20 °C to prepare serial transverse sections. Sections were stained with myofibrillar adenosine triphosphatase (ATPase) to categorize the muscle fiber type, with succinate dehydrogenase (SDH) to determine the level of mitochondrial oxidative capacity, and with alkaline phosphatase (AP) to visualize capillaries.

For ATPase staining, the sections were preincubated in barbital acetate buffer (pH 4.3) for 3 min at room temperature. After washing in 0.1 M barbital buffer containing 0.18 M CaCl\textsubscript{2} (pH 9.4) for 30 s, the sections were incubated in 0.1 M barbital buffer containing 0.18 M CaCl\textsubscript{2} and 4 mM ATP (pH 9.4) for 45 min at room temperature. The sections were then washed in 1% CaCl\textsubscript{2} with 2% CoCl\textsubscript{2} added for 3 min, and finally washed in 0.01 M sodium barbital. After washing with distilled water, the sections were visualized using 1% ammonium sulfide.

For SDH staining, the sections were incubated in 0.05% nitro blue tetrazolium and 0.05 M sodium succinate in 0.05 M phosphate buffer (pH 7.5) for 45 min at 37 °C. For AP staining, the sections were incubated in 0.1% 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt and 0.1% nitro blue tetrazolium in 0.2 M borate buffer for 45 min at 37 °C, and then fixed with 4% paraformaldehyde. The sections were observed under a light microscope (BX-51, Olympus, Japan), and images were captured using a CCD camera (VB-7010, Keyence, Japan).

The cross-sectional area and SDH activity of muscle fiber, capillary-to-muscle fiber ratio, and number of capillaries around the muscle fibers were measured and calculated using the histological images. The values were determined from 4 images, two of the deep layer and two of the superficial layer. Each muscle fiber was examined for ATPase and SDH staining and categorized as slow-oxidative (type I) fast-oxidative (type IIA) or fast-glycolic (type IIB) fibers. Muscle fibers found to be darkly stained with ATPase were categorized as type I. Muscle fibers lightly stained with ATPase and darkly stained with SDH were categorized as type IIA, and those lightly stained with both ATPase and SDH were categorized as type IIB. The sections stained by ATPase and SDH were used to determine the fiber type composition of the muscle, and the cross-sectional areas and SDH activity of each muscle fiber were measured. The capillary-to-muscle fiber ratio and number of capillaries around the muscle fiber were measured using the images with AP staining. At least 50 muscle fibers selected randomly in a section were analyzed to calculate the capillary-to-muscle fiber ratio and the number of capillaries around the muscle fiber. All measurements were made using the Image J software program (Ver.1.45, Windows).

The data were expressed as mean ± SEM. Significant differences were determined by one-way analysis of variance (ANOVA). When ANOVA was significant, group differences were determined using the Tukey-Kramer post hoc test. \textit{p}<0.05 was considered statistically significant.

RESULTS

Body weights and muscle wet weights are shown in Table 1. The wet weight of the plantaris muscle decreased approximately 15% after 1 week of hind limb unloading. In addition, the muscle wet weights decreased in the HA group. The body weights and muscle wet weights in the CA group were not significantly different from those in the Cont group.

Representative images of ATPase and SDH staining are shown in Figure 1 and Figure 2, respectively. The percentage of type I muscle fibers in the deep layer was 21% in the Cont group, 22% in the CA group, 19% in the HU group, and 18% in the HA group, with no significant differences among the 4 groups. Type I muscle fiber was not observed in the superficial layer of any group.

The values of the cross-sectional areas of all muscle fiber types in the deep and superficial layers are shown in Table 2. In the deep layer, the cross-sectional area values of all muscle fiber types in the HU and HA groups were significantly lower than those in the Cont group. The cross-sectional area values of all the muscle fiber types in the HA

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<th>Table 1. Body weight and muscle wet weight</th>
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Values are presented as mean ± SEM. Cont, control group; CA, control treated with astaxanthin group; HU, hind limb unloading group; HA, hind limb unloading treated with astaxanthin group.* significantly different from the Cont group, p<0.05
group were not significantly different from those in the HU group. Moreover, the cross-sectional area values of all the muscle fiber types in the CA group were not significantly different from those in the Cont group. In the superficial layer, similar tendencies were observed for all muscle fibers. The SDH activity values of muscle fibers in the deep and superficial layers are shown in Table 3. In the deep layer, the SDH activity values of all muscle fiber types in the HU
The SDH activity values of all muscle fiber types in the HA group were significantly higher than those in the HU group and were similar to the values found in the Cont group. The SDH activity values of all muscle fiber types in the CA group were significantly higher than those in the Cont group. SDH activity in the superficial layer was not significantly different among the 4 groups.

Representative images of AP staining are shown in Figure 3. The capillary-to-muscle fiber ratios are shown in Table 4. In the deep layer, the ratio in the HU group was significantly lower than that in the Cont group. The ratio in the HA group was significantly higher than that in the HU group, and the ratio was similar to that in the Cont group. The ratio in the CA group was significantly higher than that in the Cont group. In the superficial layer, there were no significant ratio differences among the 4 groups. The numbers of capillaries around the muscle fibers are shown in Table 5. The number of capillaries around the muscle fibers showed a pattern similar to the capillary-to-muscle fiber ratio.
DISCUSSION

Only the deep layer of the plantaris muscle displayed capillary regression and reduced SDH activity after hindlimb unloading for 1 week after astaxanthin administration. In contrast, capillary regression and reduced SDH activity were not observed in the superficial layers of atrophied plantaris muscle. Oxidative fibers have high mitochondrial oxidative enzyme activity\(^2\) and are predominantly located in the deep layer of the muscle\(^10\). In our present study, it was found that SDH activity was higher in the oxidative fibers (i.e., type I and IIA fibers) than in the glycolic fibers (i.e., type IIB fibers), and few oxidative fibers are located in the superficial layer of the plantaris muscle.

In general, the capillary density in the deep layer of a skeletal muscle is greater than that in the superficial layer\(^11\). Our study corroborated this finding, as greater capillary density was observed in the deep layer of the plantaris muscle. Therefore, high SDH activity of the oxidative fibers may be associated with the greater capillary density in the deep layer of the muscle. Capillary regression and reduced SDH activity were found only in the deep layer of the plantaris muscle, suggesting that capillary regression in the skeletal muscle starts around muscle fibers characterized by high mitochondrial oxidative enzyme activity. A previous study reported greater capillary regression in oxidative muscle than in glycolic muscle\(^13\). Additionally, reduced mitochondrial activity leads to capillary regression\(^11\). Because SDH activity is indicative of mitochondrial oxidative capacity\(^14\), capillary regression in the deep layer of the plantaris muscle may result from reduction in mitochondrial activity induced by unloading for 1 week.

Astaxanthin administration prevented regression of capillaries and reduced SDH activity in the deep layer of the plantaris muscle. Some studies have reported that unloading causes oxidative stress such as overexpression of reactive oxygen species, leading to capillary regression in the atrophied muscle\(^15\),\(^16\). Although elevation of reactive oxygen species was not observed in the present study, the overexpression of reactive oxygen species has been shown in similarly atrophied muscles in other studies\(^4\),\(^5\), and in the present study, atrophied plantaris muscle may have been exposed to oxidative stress. Unsaturated fatty acids, a major component of mitochondrial membrane, are oxidized by reactive oxygen species\(^17\), and astaxanthin has been shown to inhibit lipid peroxidation\(^18\). Furthermore, it has been suggested that in vitro astaxanthin protects mitochondrial membrane potential from oxidative stress\(^19\). In the present study, astaxanthin administration inhibited the reduction of SDH activity induced by unloading, suggesting that mitochondrial function was maintained by the inhibitory effect of astaxanthin on lipid peroxidation. Therefore, maintenance of mitochondrial function by astaxanthin led to a stable oxygen supply to the muscle fibers, resulting in inhibition of capillary regression in the deep layer of the plantaris muscle.

This study demonstrated that capillary regression in skeletal muscle is initiated in the deep layer, where the muscle fibers are characterized by high oxidative enzyme activity, and that capillary regression is prevented by astaxanthin administration. Previous studies have shown that astaxanthin enhances lipid metabolism and endurance\(20\),\(21\), and enhanced endurance may be associated with the capillary regression observed in the CA group, and the prevention of capillary regression observed in the HA group. Despite these findings, it is important to note that astaxanthin does not prevent muscle atrophy. Future, studies should consider looking into interventions for preventing muscle protein loss, such as exercise training.

REFERENCES

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