Protective effects of chlorogenic acid on capillary regression caused by disuse muscle atrophy

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ABSTRACT

Inactivity causes muscle atrophy and capillary regression in skeletal muscle. Chlorogenic acid has an antioxidant capacity and may prevent capillary regression. Therefore, the protective effects of chlorogenic acid on inactivity-induced capillary regression in rat soleus muscle were investigated. Twenty male Wistar rats were randomly divided into four groups: control (CON), chlorogenic acid supplementation (CGA), 2-week hindlimb unloading (HU), 2-week hindlimb unloading plus chlorogenic acid supplementation (HU+CGA). The rats in CGA and HU+CGA groups were orally administered chlorogenic acid (850 mg/kg/day). Unloading resulted in a decrease in capillary number, oxidative capacity, and an increase in oxidative stress of the soleus muscle, whereas chlorogenic acid supplementation prevented capillary and metabolic changes resulting from unloading by reducing oxidative stress. In conclusion, chlorogenic acid supplementation may qualify as an effective treatment to reduce capillary regression in skeletal muscle caused by disuse muscle atrophy.

INTRODUCTION

Capillaries play a key role in the delivery of oxygen and nutrients to skeletal muscle and are of significant plasticity (Olfert and Birot 2011; Fujino et al. 2014). The capillary network in skeletal muscle changes in response to physiological or pathological conditions. Long-term inactivity results in not only decreased muscle fibers, but also capillary regression in skeletal muscle (Kanazashi et al. 2014, 2019; Kanazawa et al. 2014). The capillary regression in skeletal muscle is most likely to drop fatigue resistance, since capillaries are associated with exercise capacity (Fujino et al. 2005). Therefore, it is necessary to prevent capillary regression induced by inactivity.

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Decreased physical activity leads to muscle atrophy due to the increased oxidative stress, and oxidative stress signaling is associated with conditions such as proteolysis, apoptosis, capillary regression and reduced oxidative capacity (Jackman and Kandarian 2004; Andrianjafiniony et al. 2010; Wagatsuma et al. 2011; Fujino et al. 2014). Hindlimb unloading also induces overproduction of reactive oxygen species (ROS) associated with oxidative stress (Servais et al. 2007). Thus, ROS production in inactive skeletal muscle may play an important role in muscle atrophy associated with the decreased neuromuscular activity. Moreover, antioxidant supplementation has been shown to be an effective strategy to prevent capillary regression in previous studies (Kanazashi 2013; Powers 2014), and its effects were achieved by modulating the balance between pro-angiogenesis and anti-angiogenesis factors, such as vascular endothelial growth factor (VEGF) and thrombospondin-1. In addition, muscle atrophy resulted in a decrease in VEGF protein in skeletal muscle, whereas antioxidant supplementation caused an increase in VEGF expression, which suggested
that the blood vessel growth and the delivery of oxygen and nutrients would be promoted (Kanazashi 2013). These effects may suggest that antioxidant treatment plays a crucial role in alleviating capillary regression in skeletal muscle.

Chlorogenic acid (CGA), as a family of esters formed between quinic and cinnamic acids, which represents an important group of phenolic plant secondary metabolites, produced by certain plant species and is highly absorbed and metabolized in the human body (Clifford 1999; Farah et al. 2008). The main source of CGA is green coffee beans, and CGA is also an essential component of traditional medicines such as Lonicera japonica and Eucommia bark (Tajik et al. 2017). A series of health benefits have been reported associated with CGA such as antioxidant, anti-inflammatory, and anti-platelet effects (Naveed et al. 2018; Clifford et al. 2020).

Based on the above studies, we hypothesized that chlorogenic acid supplementation would prevent capillary regression caused by muscle atrophy. In our investigation, the duration of the hindlimb unloading (HU) is set for 2 weeks as the dynamic changes in capillaries and molecules start to occur during the first week of HU (Kano et al. 2000).

MATERIALS AND METHODS

Experimental groups. Twenty male Wistar rats (402 ± 4 g) were purchased from Japan SLC (Hamamatsu, Japan). After one week of acclimatization, rats were divided into the following four groups: control (CON), chlorogenic acid supplementation (CGA), hindlimb unloaded (HU), hindlimb unloaded plus chlorogenic acid supplementation (HU+CGA). This study was approved by the Institutional Animal Care and Use Committee and was performed according to the Kobe University Animal Experimentation Regulations. All experiments were conducted in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (National Research Council 1996).

Hindlimb unloading. Hindlimb unloading was carried out in a modified method based on the previously described procedure (Morey 1979) with modification. In brief, every rat was suspended with tail by an adhesive tape and a kite string so as to prevent its hindlimb from bearing on the floor or sides of a cage. Their forelimbs were allowed to maintain contact on the floor of the cage. All rats were housed in an isolated and environmental-controlled room with a temperature of 22 ± 2°C, a light-dark cycle of 12 – 12 hours and were fed food and drinking water ad libitum.

Chlorogenic acid supplementation. The chlorogenic acid (content: approximately 40%) used in the experiment was decaffeinated from green coffee beans through a supercritical CO2 extraction system and subsequently extracted with ethanol (UCC Ueshima Coffee Co. LTD., Japan). The rats in CGA and HU+CGA groups were orally administrated chlorogenic acid dissolved in distilled water twice a day (850 mg/kg/day, with an 8-hour interval between the two doses). Rats in other groups were orally administered distilled water. After 4 days of administration for acclimatizing, all rats were administrated distilled water or chlorogenic acid during HU.

Muscle preparation. About 24 hours after the final oral administration, all rats were deeply anesthetized due to inhalation of 4% isoflurane and killed by intraperitoneal administration of sodium pentobarbital (100 mg/kg). Soleus muscles were removed and weighed and then immediately stored in a dry ice acetone bath and until −80°C prior to analysis.

Histological analysis. Transverse soleus muscle sections of 10 μm thickness were cut on a cryostat (CM-1510S; Leica Microsystems, Mannheim, Germany) at −25°C and were mounted on glass slides. The transverse sections were stained for alkaline phosphatase (AP) to visualize capillaries; the sections were incubated in 5-bromo-4-chloro-3-indolyl phosphate (AP) to visualize capillaries; the sections were incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium for 45 min at 37°C and fixed with 4% paraformaldehyde. The capillary-to-fiber (C/F) ratio was determined by counting capillaries and myofibers on each cryosection using microscopic images of AP staining.

Sections were stained using succinate dehydrogenase (SDH) to assess the skeletal muscle oxidative capacity as previously described (Kanazashi et al. 2019). Briefly, the sections were incubated in 0.1-M phosphate buffer (pH 7.6) containing 0.9-mM NaNO3, 0.9-mM 1-methoxyphenazine methylsulfate, 1.5-mM nitroblue tetrazolium, 5.6-mM EDTA-disodium salt, and 48-mM succinate disodium salt for 45 min at 37°C. Densitometric analysis was performed to determine SDH activity in each group.

The other sections were stained for myofibrillar adenosine triphosphatase (ATPase) resulting from preincubation at pH 4.2 and pH 10.8 to identify muscle fiber type. The stained sections were used to measure fiber cross-sectional area (FCSA) and slow-twitched fiber composition.
Chlorogenic acid prevents capillary regression

These results suggested that unloading decreased the body and soleus muscle weight.

Capillary number

Representative images of the stained capillary from soleus muscles are shown in Fig. 1A–D. The C/F ratio in the HU groups was significantly lower than that in the CON and CGA groups. The C/F ratio was significantly higher in the CGA group than in the CON group. Furthermore, the ratio in the HU+CGA group was significantly higher than that in the HU group (Fig. 1E). These results indicated that HU harmed the capillary network while CGA treatment had a positive effect on the capillary network.

Oxidative enzyme activity

Representative images of SDH staining in each group are shown in Fig. 2A–D. The SDH activity was significantly higher in the CGA, HU, and HU+CGA groups than in the CON group, and the value in the HU+CGA group was significantly higher than that in the HU and CGA groups (Fig. 2E). The integrated SDH activity, which reflects the total mitochondrial enzymes, in contrast, was significantly lower in the HU and HU+CGA groups than in the CON and CGA groups. The integrated SDH activity in the CGA group was significantly higher compared to that in the CON group, whereas the integrated SDH activity in the HU+CGA group was significantly higher than that in the HU group (Fig. 2F). These results demonstrated that mitochondrial oxidative activity was reduced in HU and improved in CGA treatment.

Fiber cross-sectional areas and slow-twitch fiber composition

Representative images of ATPase staining for each group are shown in Fig. 3A–H. The fiber cross-sectional area (FCSA) of soleus muscle was significantly lower in the HU and HU+CGA groups compared

In situ detection of reactive oxygen species (ROS) generation was evaluated with the fluorescent probe dihydroethidium (DHE) which detects oxidative stress by glowing after interacting with O$_2^-$ to form oxyethidium. This staining method has been used previously to determine ROS activity in skeletal muscles (Bouitbir et al. 2011; Cozzoli et al. 2011). DHE is cell-permeable and interacts with nucleic acids to emit a bright color detectable qualitatively by fluorescent microscopy (model BX51; Olympus, Tokyo, Japan). Briefly, the transverse sections were incubated with 5 × 10$^{-6}$ mol/L DHE (Wako Pure Chemicals, Osaka, Japan) for 30 min at 37°C in the dark box, following which the sections were rinsed with 37°C phosphate-buffered saline (PBS) and observed under a fluorescent microscope equipped with a filter (excitation at 545 nm). The fluorescence intensity was determined by the fluorescence area fraction using grayscale analysis in four images per muscle.

All measurements were performed utilizing the NIH ImageJ software program (NIH, Bethesda, MD, USA).

Statistical analysis. All data are expressed as means ± standard error of the mean (SEM). One-way ANOVA was used to determine overall significant differences between groups, and then Tukey’s post hoc test was performed to determine specific group differences. For all tests, the significance level was set at $P < 0.05$.

RESULTS

Body mass, soleus muscle and relative muscle weight

The body mass, soleus muscle and relative muscle weight were significantly lower in the HU and HU+CGA groups than in the CON and CGA groups. However, there was no statistical significance between the HU and HU+CGA groups (Table 1).

|                | Body weight (g) | Soleus muscle weight (mg) | Relative soleus muscle weight (mg/100 g) |
|----------------|-----------------|---------------------------|-----------------------------------------|
| CON            | 392.4 ± 14.0    | 171.2 ± 7.7               | 43.7 ± 1.4                              |
| CGA            | 386.2 ± 6.7     | 153.2 ± 6.5               | 39.6 ± 1.2                              |
| HU             | 328.0 ± 8.2*†   | 112.4 ± 5.4*†             | 34.2 ± 1.1*†                            |
| HU+CGA         | 339.6 ± 4.2*†   | 105.0 ± 3.9*†             | 30.9 ± 0.9*†                            |

Values are means ± SEM (n = 5). Abbreviations: CON, control; CGA, chlorogenic acid; HU, hindlimb unloaded; HU+CGA, hindlimb unloaded plus chlorogenic acid. The symbols * and † indicate significant differences from the CON and CGA groups, respectively, at $P < 0.05$. Unloading (HU and HU+GGA) resulted in a decrease in the body and soleus muscle weight.

Table 1

These results suggested that unloading decreased the body and soleus muscle weight.
Fig. 1 Transverse sections stained for alkaline phosphatase (A–D) and C/F ratio (E) of the soleus muscles. CON (A), CGA (B), HU (C), HU+CGA (D). Capillaries were visualized as black dots. Bar = 100 μm. Values are means ± SEM (n = 5). Abbreviations: CON, control; CGA, chlorogenic acid; HU, hindlimb unloaded; HU+CGA, hindlimb unloaded plus chlorogenic acid. The symbols *, † and ‡ indicate significant differences from the CON, CGA and HU groups, respectively, at P < 0.05. HU harmed the capillary network while CGA treatment prevented the capillary regression in the skeletal muscle.

Fig. 2 Transverse sections stained for succinate dehydrogenase (SDH) (A–D), SDH activity (E) and Integrated SDH activity (F) of the soleus muscles. CON (A), CGA (B), HU (C), HU+CGA (D). Bar = 100 μm. Values are means ± SEM (n = 5). Abbreviations: CON, control; CGA, chlorogenic acid; HU, hindlimb unloaded; HU+CGA, hindlimb unloaded plus chlorogenic acid. The symbols *, † and ‡ indicate significant differences from the CON, CGA and HU groups, respectively, at P < 0.05. The activity of mitochondrial oxidase was reduced in HU and improved by CGA treatment.
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Fig. 3 Transverse sections stained for adenosine triphosphate (ATPase) (A–H, Left pH: 4.2; Right pH: 10.8) and fiber cross sectional area (I) and slow fiber composition (J) of the soleus muscles. CON (A; B), CGA (C; D), HU (E; F), HU+CGA (G; H). Bar = 100 μm. Values are means ± SEM (n = 5). Abbreviations: CON, control; CGA, chlorogenic acid; HU, hindlimb unloaded; HU+CGA, hindlimb unloaded plus chlorogenic acid; a: type I; b: type IIa; c: type IId/x. The symbols *, † and ‡ indicate significant differences from the CON, CGA and HU groups, respectively, at P < 0.05. CGA treatment attenuated the HU-induced reduction of FCSA and caused a slow to fast muscle type transformation.

Production of ROS
Intracellular ROS production was measured by DHE fluorescence in the nucleus. The representative images of the soleus muscle stained with DHE in each group are shown in Fig. 4A–D. The DHE fluorescence intensity in the HU group was significantly higher than that in the CON, CGA, and HU+CGA groups. On the contrary, the value in the HU+CGA group was significantly lower than that in the HU group (Fig. 4E). These results revealed that ROS were higher in HU, but CGA treatment could reduce the overproduction of ROS.

DISCUSSION
The novel findings of this study were as follows: (1) capillary regression was observed in the soleus muscle of hindlimb-unloaded rats, which was accompanied by the overexpression of ROS; (2) chlorogenic acid treatment attenuated muscle atrophy and capillary regression, as well as reduced mitochondrial oxidative capacity by reducing the overproduction of oxidative stress. Based on the results, we are convinced that chlorogenic acid treatment is an effective treatment for both muscle atrophy and capillary regression in unloaded skeletal muscle.
In the previous studies, two-week HU resulted in soleus muscle atrophy and capillary regression reflected by decreased muscle mass, fiber cross-sectional area, C/F ratio, and slow-to-fast isoforms fiber transition (Kanazashi et al. 2014). Consistent with previous studies, our study showed that HU induced capillary regression in unloaded soleus muscle. Kondo et al. (1993) have reported that hindlimb unloading induced an increase in oxidative stress in hindlimb muscles due to ROS overexpression, which damaged mitochondria leading to mitochondrial dysfunction (Kanazashi et al. 2014). Additionally, oxidative stress associated with unloading is involved in capillary regression within skeletal muscle (Wagatsuma et al. 2011; Powers et al. 2012). Furthermore, SDH, a mitochondrial rate-limiting enzyme in the TCA cycle, is decreased when muscle activity was inhibited by hindlimb unloading (Kanazawa et al. 2014). In the present study, we observed that unloading resulted in an increase in oxidative stress from the fluorescence intensity of DHE and a decrease in SDH activity. Thus, our findings suggest that unloading induced muscle atrophy and capillary regression in soleus muscle via increased oxidative stress.

Chlorogenic acid, as one of the polyphenols, prevented the overexpression of ROS in the soleus muscle through the sirtuin 1 (SIRT1) pathway and Nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (Shi et al. 2018; Yao et al. 2019). As supported in this study, chlorogenic acid treatment reduced the overproduction of ROS, prevented unloading-induced capillary regression, and had a protective effect on the hindlimb unloading-related decrease in muscle mass. This may be related to peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) levels in the soleus muscle. Zhou et al. (2016) reported that chlorogenic acid could increase the level of mitochondrial oxidative phosphorylation through the increased expression of PGC-1α, and Miura et al. (2006) showed that the overexpression of PGC-1α also caused muscle atrophy. In the present study, chlorogenic acid treatment had a positive effect on mitochondrial oxidative capacity due to oxidative stress reduction. In addition, chlorogenic acid treatment shifted to type IIa fibers which have higher oxidase activity compared to type I fibers in the rat soleus muscle (Rivero et al. 1998; Chen et al. 2021). These
results showed that chlorogenic acid could increase mitochondrial oxidative activities in skeletal muscle under disuse condition.

The relationship between SDH activity and capillary-to-fiber ratio has also been demonstrated (Romanul 1965; Maxwell et al. 1980). Consistent with previous studies, this study showed that chlorogenic acid treatment during unloading had a positive effect on SDH activity and type IIa fibers, which could increase oxidative capacity in the soleus muscle. Therefore, our findings suggest that chlorogenic acid treatment has a positive effect on the capillary network of hindlimb-unloaded rats’ muscle.

A limitation of this study is that we only assessed the effect of chlorogenic acid by histochemical staining, and more studies are needed to confirm the effect of chlorogenic acid treatment on the balance of pro- and anti-angiogenesis in unloaded skeletal muscle.

In conclusion, we found that chlorogenic acid supplementation is an effective treatment to prevent capillary regression in soleus muscle induced by muscle atrophy. It is suggested that chlorogenic acid has the potential capacity to be used as a therapeutic intervention for the treatment of skeletal muscle vasculopathy caused by disuse-induced muscle atrophy.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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