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PDF issue: 2024-12-29

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(Degree)
博士（保健学）

(Date of Degree)
2016-03-25

(Date of Publication)
2018-03-25

(Resource Type)
doctoral thesis

(Report Number)
甲第6619号

(URL)
https://hdl.handle.net/20.500.14094/D1006619

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博 士 論 文

Abnormalities in three-dimensional capillary architecture and imbalance between vascular endothelial growth factor-A and thrombospondin-1 in soleus muscle of ovariectomized rat

（卵巣摘出がラットヒラメ筋における 3 次元毛細血管構造及び VEGF-A と TSP-1 の発現バランスに与える影響）

平成 28 年 1 月 18 日

神戸大学大学院保健学研究科保健学専攻

田中雅侑

Abnormalities in three-dimensional capillary architecture and imbalance between vascular endothelial growth factor-A and thrombospondin-1 in soleus muscle of ovariectomized rat

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Abstract:

Reduced ovarian hormone levels associated with menopause or ovariectomy (OVX) not only result in vascular dysfunction but also lead to structural abnormalities in capillaries. Therefore, the effect of OVX on the three-dimensional (3-D) architecture of capillary networks and the underlying molecular mechanisms were investigated in rat soleus muscle. Seven-week-old female Wistar rats were divided into the OVX and sham-treated (Sham) groups. The OVX group exhibited lower endurance exercise capacity compared to the sham group and resulted in decreased capillary diameter, the number of anastomoses and capillary / anastomosis volume in soleus muscle, indicating 3-D structural abnormalities of capillary networks. Furthermore, OVX led to increased concentrations of thrombospondin-1 (TSP-1) protein and a decreased VEGF-A/TSP-1 ratio, an indicator of angio-adaptations, in soleus muscle compared with the Sham group. These results indicate OVX may induce 3-D capillary regression in soleus muscle through an imbalance between VEGF-A and TSP-1 expression, possibly associated with decreased exercise tolerance in ovariectomized rats.

Keywords:

ovariectomy, skeletal muscle, capillary network, three-dimensional imaging, vascular endothelial growth factor, thrombospondin-1

1. Introduction

Decreased levels of ovarian hormones have been reported to be frequently associated with vascular-related diseases in postmenopausal women [1]. In the context of this, a decrease in ovarian hormones has been shown to seriously affect microvasculature in brain and heart. In experimental rodent models of the postmenopausal state, ovarian hormone deficiency induced by ovariectomy (OVX) results in abnormalities in capillaries of the brain [2], heart [3, 4] and dura mater [5, 6]. As ovarian hormones circulate through the capillaries of the entire body, a decrease in ovarian hormones may also impact on the microvasculature of the other peripheral organs or tissues, such as skeletal muscle. Several studies suggested a reduced ovarian hormone due to menopause was closely associated with the development of peripheral arterial disease [7-9], which limits the function of skeletal muscle owing to abnormalities of muscle microvasculature [10]. However, the influence of ovarian hormone loss due to OVX on the microvasculature of skeletal muscles remains largely unknown.

The capillaries of skeletal muscle play an important role in supplying oxygen and nutrients to and removing waste products from muscle cells. Therefore, abnormalities of muscle microvasculature can lead to a loss of functional capillaries, resulting in a decrease in the maximal capacity for delivery of oxygen and substrates to muscle cells. A number of studies suggested that a decrease in muscle capillarity has been shown to seriously impair endurance capacity [10-13] Furthermore, muscle capillarity has been shown to determine exercise tolerance also in most if not all cardiovascular diseases, including heart failure and peripheral arterial disease [10, 14-16].

Muscle capillarity is susceptible to structural change in response to various conditions. Exercise lead to expansion of the capillary bed [17], while disuse [18, 19] and diabetes [20] contributes to abnormal alteration of that. Capillary remodelling in skeletal muscle reveals not only cross-sectional changes in the number of capillaries but also three-dimensional (3-D) structural alterations of capillary networks, *i.e.* capillary volume, diameter, tortuosity and the number of anastomoses [18]. In addition, 3-D capillary alteration is not always accompanied by a decrease in the number of capillaries observed in tissue cross-sections [21]. Hence, the analysis of 3-D capillary architecture is thought to be more useful approach for elucidating relative changes in muscle capillarity associated with a variety of conditions relative to traditional methods based on two-dimensional analysis of muscle capillarity. To date, no information is available about the effect of OVX on the 3-D architecture of microvasculature in skeletal muscle. Therefore, in the present study, we used 3-D analysis to visualize structural alterations in the capillary network of skeletal muscles in OVX animals.

Capillary architecture is tightly regulated by pro- and anti-angiogenic factors in skeletal muscle. Vascular endothelial growth factor-A (VEGF-A) plays an important role in angiogenesis [13, 17, 22], whereas thrombospondin-1 (TSP-1) plays a role in repressing capillary growth owing to anti-proliferative and pro-apoptotic effects [1, 12, 23-25]. A key role for these factors has been well reported in the alterations of muscle capillaries in response to a variety of conditions including exercise [12, 13], peripheral arterial disease [26, 27], disuse [19, 28] and diabetes [21, 29]. As yet, little is known about the effect of OVX on the expression of pro-angiogenic VEGF-A and anti-angiogenic TSP-1 in skeletal muscle.

Therefore, the aim of the present study was to investigate changes in the 3-D architecture of capillaries and associated molecular mechanisms, in the soleus muscle of OVX rats, an experimental model of ovarian hormone-deficient women.

2. Materials and Methods

2.1. Animals and protocol

Seven-week-old female Wistar rats (Japan SLC, Shizuoka, Japan) weighing 137–157 g were used. The animals were randomly divided into 2 groups: sham-operated group

(Sham; n = 7) and OVX group (n = 7). Bilateral ovariectomy was performed dorsally under anaesthesia (sodium pentobarbital, 50 mg/kg, *i.p.*). Sham operations were performed by exteriorizing the ovaries. All animals were housed at a temperature of 22 ± 2°C with a 12–12 h light-dark cycle and provided standard rodent chow and water *ad libitum*. Exercise tolerance was assessed 28 weeks after surgery using a treadmill exercise test. Two weeks after the test, animals were sacrificed and the soleus muscle was excised for subsequent measurements. This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations. All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

2.2. Treadmill exercise test

Treadmill exhausting test was performed to measure maximal running endurance capacity. Rats were trained over the course of a week, 5 min/day at a constant speed of 10 m/min with no slope to run on an adapted motor-driven treadmill designed for rats. After adapting to the treadmill, the rats ran on a treadmill with a slope of 20° at a constant speed of 15 m/min until exhaustion [30]. Stainless-steel grids provided an electrical stimulus that encouraged running. Exhaustion was established when rats accepted 3 consecutive electrical stimuli instead of running. Blood lactate levels were measured from tail vein blood samples using Lactate Pro (Arkley, Kyoto, Japan) before and after the treadmill endurance test.

2.3. Tissue extraction

After 30 weeks, animals were anaesthetized with sodium pentobarbital (50 mg/kg, *i.p.*), and heparin (5,000 IU/kg, *i.p.*) was injected. Animals were placed on a heated surgical table, laparotomy was performed, and the uterus and associated white adipose tissue were removed and weighed after connective tissue was dissected. Subsequently, the left soleus muscle was removed, blotted dry and weighed. Muscle tissue was mounted on cork, rapidly frozen in an isopentane cooled in dry ice and stored at −80°C until histological and biochemical analyses. Then, for 3-D imaging of soleus capillary architecture, the right soleus muscle was perfused with a contrast medium consisting of 1% fluorescent material (Tombow, Tokyo, Japan), 8% gelatin (Nacalai Tesque, Kyoto, Japan) and distilled water using an established procedure described in previous studies [18-20]. After the animals were sacrificed, the entire body of the rat was immersed into cold saline for 15 min. Thereafter, the right soleus muscle was removed, blotted dry, mounted on cork of near-physiological length, rapidly frozen in isopentane cooled in dry ice and stored at −80°C until analyses in 3-D architecture of capillary network.

2.4. Histochemical analysis

Left soleus muscles were sliced into 12-μm thick transverse sections using a cryostat microtome (CM-1510S, Leica Microsystems, Mannheim, Germany). Sections were stained with alkaline phosphatase (AP) to visualize capillaries in the soleus muscle. Sections were incubated in 0.1% 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium for 45 min at 37°C and fixed with 4% paraformaldehyde, as previously described [28, 31]. The mean fibre cross-sectional area was determined for 300–500 fibres per muscle using microscopic images of AP staining. The capillary density was measured by counting all capillaries in a 1-mm^2 muscle cross-section. All measurements were performed using NIH ImageJ software program (NIH, Bethesda, MD, USA).

2.5. Three-dimensional visualization of the capillary network

To visualize 3-D capillary architecture in soleus muscle, a confocal laser scanning microscope (C2, Nikon, Tokyo, Japan) with an argon laser (488 nm) was used, as previously described [18-20]. Briefly, the sample block of the right soleus muscle was longitudinally sliced into 100 μm sections using a cryostat (CM-1510S, Leica Microsystems, Mannheim, Germany). Microscopic 3-

D capillary images were obtained at a magnification of 20× and scanned in 1-μm thick longitudinal slices over a total depth of 50 μm. To visualize the 3-D architecture of capillary networks, the 50 files were stacked and converted into digital images. Using 3-D images of capillary networks, capillary diameter, the number of anastomoses and capillary / anastomosis volume were measured in volume of 2 mm^3 (200 μm length, 200 μm width and 50 μm depth) using NIH ImageJ software (NIH, Bethesda, MD, USA). All measurements were performed using 7–10 stacked images per muscle.

2.6. Citrate synthase and β-hydroxyacyl-CoA dehydrogenase activities

Citrate synthase (CS) activity was measured from the left soleus muscle. The sample was homogenized in 10-mM Tris (pH 7.4), 175-mM KCl and 2-mM EDTA. Homogenates were frozen and thawed thrice and then centrifuged at 15,000*g* for 10 min at 4°C. Supernatants were collected and used for the measuring the CS activity by Srere's method [32]. Briefly, supernatants were reacted with 5-mM oxaloacetate after addition of 100-mM Tris (pH 7.4), 3-mM acetyl-CoA, 1-mM 5, 5'-dithobis [2-nitrobenzoric acid] (DTNB) and absorbance measured at 412 nm for 5 min.

β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity was measured from samples prepared for the CS assay. Measurements were performed using Bass's method [33]. Briefly, supernatants were reacted with 0.1-mM acetoacetyl-CoA after addition of 167-mM triethanolamine (pH 7.4), 50-mM EDTA and 0.3-mM NADH, and absorbance measured at 340nm for 5 min.

2.7. Western blotting

Portions (approximately 20 mg) of each left soleus muscle were homogenized in RIPA lysis buffer containing 1-mM Na_3VO_4, 1-mM NaF and protease inhibitor cocktail (1:100, P8340; Sigma Chemicals, Perth, WA, USA). Total supernatant protein concentrations were determined according to Bradford method using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) before loading onto either 7.5% (40 μg/lane) or 12.5% (20 μg/lane) SDS-polyacrylamide gels. Proteins were blotted on polyvinylidene difluoride (PVDF) membranes and blocked for 1 h with 5% skimmed milk in PBS with 0.1% Tween 20 (PBST). Membranes were incubated using antibodies against VEGF-A (1:200 in PBST, sc-7269; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or TSP-1 (1:100 in PBST, sc-59887; Santa Cruz Biotechnology) overnight at 4°C and then incubated in a solution with HRP-conjugated anti-mouse (1:1000 in PBST, GE Healthcare, Waukesha, WI, USA) for 1 h. Proteins were detected using EzWestLumi One (ATTO, Tokyo, Japan). Finally, images were analysed with a LAS-1000 (Fujifilm, Tokyo, Japan) using a chemiluminescent image analyzer and quantified using the Multi-Gauge Image Analysis Software program (Fujifilm) against a relative concentration of β-actin (1:1000 in PBST, sc-47778; Santa Cruz Biotechnology) as an internal control. The used primary antibodies, i.e. VEGF-A, TSP-1 and β-actin, in this study were appropriate for western blot and validated to be used with the experimental subjects in the previous studies [31, 34]. VEGF-A-to-TSP-1 (VEGF-A/TSP-1) ratios were calculated and used to reflect the balance between the levels of pro- and anti-angiogenic factors [28].

2.8. Statistical analysis

All values were expressed as mean and SEM. Significant differences in blood lactate levels in each group between before and after exercise testing were analysed by the paired Student's *t* test. Significant differences between Sham and OVX groups were analysed by unpaired Student's *t* test. Results were deemed statistically significant level at $p < 0.05$.

3. Results

3.1. Maximal endurance running capacities and plasma lactate levels

The maximum running endurance time in the OVX group was significantly lower than that in the Sham group (Fig. 1A). There was no difference in plasma lactate

Table 1
Body weight, visceral fat weight and uterus weight

	Initial body weight (g)	Final body weight (g)	Peri-uteral adipose tissue weight (g)	Uterus weight (mg)
Sham	143 ± 3	238 ± 4	8.7 ± 0.3	539 ± 36
OVX	143 ± 2	294 ± 7*	10.5 ± 0.3*	80 ± 8*

Values indicate means ± SEM (n = 7 in each group). The symbol * is significantly different from the Sham group, at p <0.05.

levels before the running test between the Sham and OVX groups, but those in both the Sham and OVX groups showed a significant increase after the running test. Furthermore, the plasma lactate level after the running test in the OVX group was significantly higher than that in the Sham group (Fig. 1B).

3.2. Body weight, visceral fat mass and uterus mass

Final body weights were significantly higher in the OVX group than those in the Sham group (Table 1). In this study, peri-uteral adipose tissue and uterus were harvested to estimate the efficiency of the ovariectomy to reduce ovarian hormone levels. Peri-uteral adipose tissue weights were significantly higher in the OVX group than in the Sham group (Table 1), whereas uterus weights in the OVX group were significantly lower than those in the Sham group (Table 1).These results coincide with the previous studies suggesting that significant visceral fat gain and uterus atrophy are induced by OVX [35-37].

3.3. Soleus muscle mass, fibre cross-sectional area and capillary density

No difference in soleus muscle mass or fibre cross-sectional area was observed between the Sham and OVX groups (Table 2). Additionally, the ratio of capillary density to muscle cross-sectional area was similar in both groups (Table 2).

3.4. Muscle citrate synthase and β-hydroxyacyl-CoA dehydrogenase activities

CS and β-HAD are the key mitochondrial enzymes in the tricarboxylic acid cycle and β-oxidation of fatty acids, respectively, associated with the oxidative capacity of skeletal muscle. There was no significant difference in CS activity between the two groups (Table 2). However, β-HAD activity was significantly higher in the OVX group than in the Sham group (Table 2).

3.5. Capillary diameter, the number of anastomoses and capillary / anastomosis volume

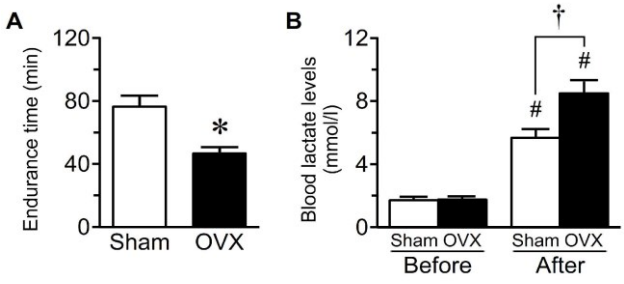

Fig. 1. The maximal endurance time (A) and the blood lactate levels before and after the treadmill exhausting test (B). The values indicate means ± SEM (n = 7 in each group). The symbol # is significantly different from the lactate level before the test in the each group, and the symbol † is significantly different from the lactate level after the test in the Sham group, respectively, at p <0.05. The following abbreviations are used for Figures 1–3: Sham, sham operated; OVX, ovariectomized group.

Table 2
Soleus wet weight, fibre cross-sectional area, capillary density and mitochondrial enzyme activities

	Soleus wet weight (mg)	Fibre cross-sectional area (μm^2)	Capillary density (no./mm^2)	CS activity (nmol/min/mg)	β-HAD activity (nmol/min/mg)
Sham	94 ± 4	2932 ± 120	631 ± 13	179 ± 8	66 ± 3
OVX	106 ± 5	3276 ± 153	649 ± 25	191 ± 13	77 ± 3*

Values indicate means ± SEM (n = 7 in each group). The symbol * is significantly different from the Sham group, at $p < 0.05$.

The 3-dimensional capillary architecture was analysed using a confocal laser-scanning microscope as described previously [18-20]. Representative confocal images of soleus muscle capillaries and intercapillary anastomoses from each group are shown in Fig. 2A and B. The histogram of luminal capillary diameter was shown in Fig. 2C and those in the OVX group was shifted towards reduced capillary diameter compared to that of the Sham group, indicating that smaller capillaries were more abundant in the OVX group. The mean capillary diameter, number of anastomoses and capillary / anastomosis volume in the OVX group were significantly lower than those in the Sham group (Fig. 2D-F).

3.6. Expression levels of VEGF-A and TSP-1 proteins and the VEGF-A/TSP-1 ratio

Representative western blots for VEGF-A and TSP-1 in soleus muscle from Sham and OVX groups are shown in Fig. 3A and B. No significant difference in the amount of VEGF-A protein was observed between the Sham and OVX groups (Fig. 3A). The amount of TSP-1 protein, however, was significantly higher in the OVX group than in the Sham group (Fig. 3B). Consequently, the VEGF-A/TSP-1 ratio was significantly lower in the OVX group than in the Sham group (Fig. 3C).

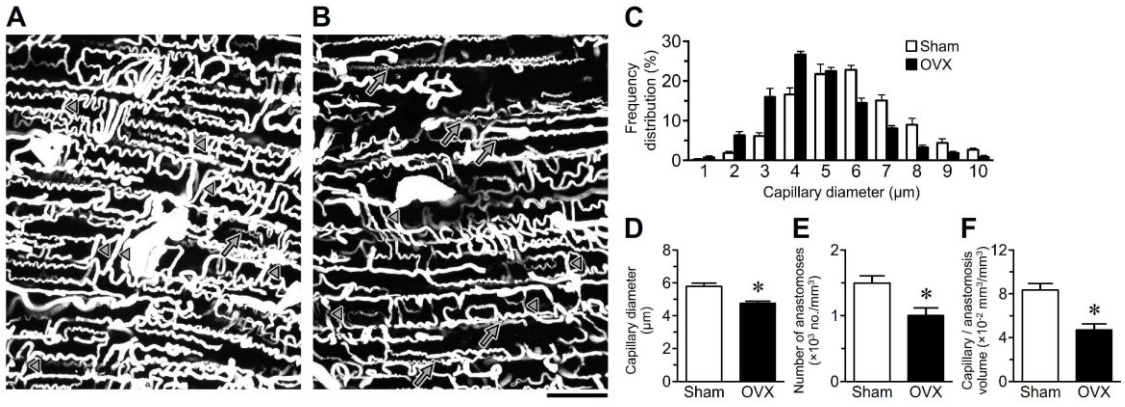

Fig. 2. Representative images of the capillaries and intercapillary anastomoses architecture of the soleus muscle in the Sham (A) and OVX (B) group. Arrows indicate capillaries with a diameter of <2.5 μm and arrowheads show intercapillary anastomoses. Image depth (z-axis) = 50 μm; scale bar represents 100 μm. C: Frequency distribution of the luminal diameter of the capillaries in the soleus muscles in the Sham and OVX group. Also shown are the mean capillary diameter (D), number of anastomoses (E) and capillary / anastomosis volume (F). These measurements were determined using a confocal laser scanning microscope. The values indicate means ± SEM (n = 7 in each group). The symbol * is significantly different from the Sham group, at $p < 0.05$.

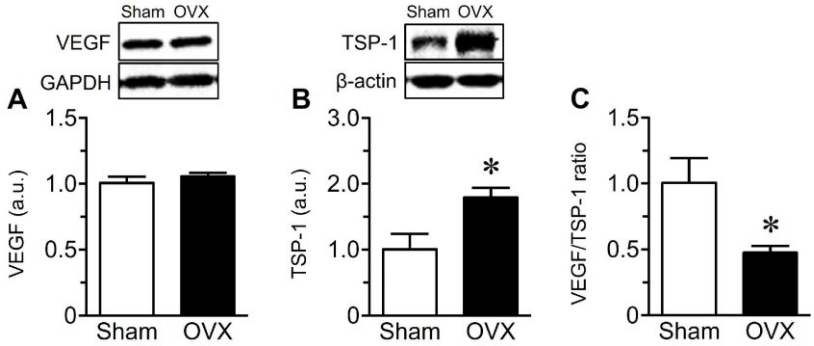

Fig. 3. Protein levels of VEGF-A (A), TSP-1 (B) and the VEGF-A/TSP-1 ratio (C) in the Sham and OVX groups. Each protein was quantified in relation to the levels of β-actin proteins from each gel. The expression levels are calculated as the fold change (in a.u.) relative to the Sham group and indicate means ± SEM (n = 7 in each group). The symbol * is significantly different from the Sham group, at $p < 0.05$.

4. Discussion

In the present study, we found a decrease in capillary diameter, the number of anastomoses and capillary / anastomosis volume of the soleus muscle in the OVX group using 3-D capillary network observation. Furthermore, the VEGF-A/TSP-1 ratio was lower in the OVX group compared with that in the Sham group due to an increase in TSP-1 expression in soleus muscle. These observations indicate that OVX may induce capillary rarefaction in skeletal muscle due to an associated imbalance between pro-angiogenic VEGF-A and anti-angiogenic factor TSP-1 levels.

It has been previously reported that exposure to ovarian hormones can promote angiogenesis [38], while there is little information about the effect of ovarian hormone loss on angio-adaptation in skeletal muscle of ovariectomized rats. A number of studies have reported that OVX-induced muscle impairment results from a deficiency in circulating ovarian hormones [37, 39, 40]. In the OVX rodent model, ovarian hormone deficiency has been reported to induce an increase in body and visceral fat mass [35-37]. In addition, uterus atrophy was observed and used as an indicator of ovarian hormone deficiency in a previous study [41]. Similarly, our results demonstrated increased body and visceral fat mass and decreased uteral mass in the OVX group, indicating that the OVX group had ovarian hormone deficiency in this study.

Muscle capillarity is susceptible to structural change in response to various conditions. Structural change in capillary networks is measured by changes in morphological properties including not only capillary number but also in capillary diameter, volume, tortuosity and the number of anastomoses. In the present study, the OVX group had decreased capillary diameter, number of anastomoses and capillary / anastomosis volume without a decrease in the number of capillaries when compared with those in the Sham group. Therefore, this study demonstrates for the first time that OVX has an impact of the microvasculature of the soleus muscle.

Skeletal muscle capillaries are necessary for oxygen supply to muscle cells and to determine oxygen supply capacity. Therefore, capillary structural alteration in skeletal muscle leads to affect oxygen supply to muscle cells. The decreased number of muscle capillaries, followed by a possibly reduced muscle perfusion during exercise, has been shown to be closely related with exercise intolerance [10-13]. Similarly, the other characters of microvasculature, including capillary diameter, anastomosis number and capillary / anastomosis volume, may also have an impact on muscle perfusion during exercise as well as the number of muscle capillaries and, additionally, exercise tolerance. Abnormalities of microvasculature were observed as a decrease in the capillary diameter, anastomoses number and capillary / anastomosis volume not in

the capillary number in soleus muscle of the OVX group in this study. In addition, capillaries with a diameter of <2.5 μm, which were preventing erythrocyte flow [42], were observed more frequently in the OVX group than in the Sham group, suggesting that a higher percentage of capillaries in the OVX group were unavailable for oxygen supply than that in the Sham group. These alterations led to a loss of functional capillaries in soleus muscle of the OVX group, possibly contributing to a smaller oxygen supply to muscle cells during the maximal exercise test relative to the Sham group. Indeed, the OVX group exhibited a decrease in exercise endurance time and an increase in blood lactate levels after the exercise test in this study. On the other hand, CS activity, an indicator of mitochondrial oxidative capacity, did not differ between the Sham and OVX groups. This result is consistent with previous studies showing that muscle CS activity is unchanged between sham- and OVX-treated animals [37, 40, 43]. β-HAD activity in soleus muscle as an indicator of β-oxidation was also investigated. Surprisingly, β-HAD activity in the OVX group was higher than that in the Sham group. Some studies have shown that OVX decreases β-oxidation-related enzymatic activities in skeletal muscle [41, 43], whereas other studies failed to detect a difference in β-oxidation enzymes from sham-treated rats [40, 44, 45]. From these and previous results, there is little consensus on the adaptation of muscle β-oxidation enzymes in response to ovariectomy. Increased β-HAD activity in the OVX group may be related to ovariectomy duration in this study. In fact, 30 weeks duration of OVX is relatively long compared with previous studies. Therefore, we could not exclude the possibility that because OVX induces obesity with increased visceral fat, increased β-HAD activity in the OVX group could be accounted for by an obesity-related muscle adaptation to changes in circulating adipokines and free fatty acids [46, 47]. These observations indicate that OVX-induced capillary structural abnormalities, rather than mitochondrial alterations in skeletal muscle, may contribute to exercise intolerance and higher production of lactic acid after maximal exercise test in the OVX group. This is likely due to significant limitation of oxygen supply necessary for endurance exercise, followed by higher compensatory anaerobic metabolism.

Although muscle capillarity is often homologous with muscle mitochondrial activity, the OVX group had higher mitochondrial enzyme activity, but a reduced number in functional capillaries in soleus muscle in this present study. A previous study reported that a decrease in muscle capillarity, but an increase in muscle oxidative and mitochondrial enzyme activities were observed in muscle specific VEGF-A-deleted mice [13]. Moreover, similar observations have been reported in muscle dysfunction due to peripheral arterial disease [48, 49], chronic obstructive pulmonary disease [50] and obesity [51]. These reports indicate that muscle capillarity is not necessarily homologous with muscle mitochondrial activity. Therefore, the 3-D capillary remodelling observed in the OVX group could not be attributed to changes in muscle mitochondrial activity in this study. Since ovarian hormones have been shown to be involved in a variety of intercellular signalling pathways in skeletal muscle [36, 37, 52, 53], decreased ovarian hormone levels induced by OVX may influence microvasculature-related molecular mechanisms in soleus muscle, particularly as the OVX group exhibited greater 3-D capillary rarefaction compared to that exhibited by the Sham group.

VEGF-A is a well-known regulator of angiogenesis, stimulating the formation of new vascular networks by recruiting and promoting differentiation of endothelial cells [54]. An increase in VEGF-A expression induces angiogenesis in skeletal muscle, whereas a decrease in VEGF-A expression results in capillary rarefaction. This study found no difference in the amount of VEGF-A between the OVX and Sham groups. Alterations of muscle microvasculature are not necessarily accompanied by a decrease in VEGF-A levels under several conditions [20, 28, 31]. On the other hand, the amount of soleus muscle TSP-1 was significantly higher in the OVX group than that in the Sham group in this study. TSP-1 is commonly known as an inhibitor of angiogenesis and has an anti-proliferative and

pro-apoptotic effects [1, 12, 23-25]. An increased TSP-1 expression occurs in the skeletal muscle in the context of disuse [19, 28], diabetes [21] or peripheral arterial disease [26, 27], leading to abnormalities of the muscle microvasculature. Several studies show that an increase in TSP-1 expression in skeletal muscle induces structural alterations of microvasculature, such as decreased perimeter, diameter and volume of capillary [21, 55]. Furthermore, endothelial cell apoptosis occurs more frequently in anastomoses of muscle microvessel in the state of angiostatic response to disuse [18]. From these results, the structural alterations of muscle capillarity observed in the OVX group might result from the angiostatic effects of TSP-1. Sengupta *et al.* [56] reported that TSP-1 expression is attenuated by oestrogen in human umbilical vein endothelial cells. Therefore, higher TSP-1 expression in the OVX group in our study may be partly associated with the loss of the inhibitory effect of oestrogen on TSP-1 expression. In addition, the integrated balance between VEGF-A and TSP-1 expression in soleus muscle was measured as an indicator of muscle angio-adaptions. Consequently, a decrease in the VEGF-A/TSP-1 ratio was demonstrated in the OVX group, indicating an imbalance between pro- and anti-angiogenic factors induced by OVX. Roudier *et al.* [28] have reported that measurement of the VEGF-A/TSP-1 ratio in skeletal muscle is a more accurate approach for evaluating muscle angio-adaptations than individual analysis of the individual factors. A decrease in the VEGF-A/TSP-1 ratio is associated with abnormalities of 3-D capillary architecture in soleus muscle induced by hindlimb unloading in previous studies [19, 28]. Moreover, Kondo *et al.* [21] have demonstrated that a decrease in the VEGF-A/TSP-1 ratio contributes to decreased capillary diameter and capillary / anastomosis volume without a loss of capillary number in soleus muscle of type 2 diabetic rats. Based on these observations, decreased VEGF-A/TSP-1 ratio due to a variety of conditions can lead to a loss of functional capillaries due to 3-D capillary remodelling involving a decrease in the capillary diameter and capillary / anastomosis volume in soleus muscle. Therefore, decreased VEGF-A/TSP-1 ratio induced by OVX may affect the 3-D capillary abnormalities observed as decreased capillary luminal diameter, the number of anastomoses and capillary / anastomoses volume in the OVX group. In this study, we demonstrated the effect of OVX on levels of VEGF-A and TSP-1 in rat soleus muscle. However, we were unable to demonstrate the effects of OVX on other angiogenic factors, such as angiopoietins and VEGF-A receptors, or confirm whether ovarian hormone loss influenced signalling pathway involved in angio-adaptation. In the future, further studies using estradiol and/or progesterone replacement in OVX rats are required to determine the relationship between ovarian hormone loss due to OVX and angiogenic molecular mechanisms in skeletal muscle in greater detail.

In conclusion, this study demonstrates a novel effect of OVX on the architecture of the capillary bed. OVX induces a loss of functional capillaries, as shown by a reduced diameter and suggests that the underlying mechanism could involve an imbalance between pro-angiogenic VEGF-A and anti-angiogenic TSP-1 expression. The OVX-induced capillary alterations are likely associated with muscle perfusion that is resulting in a reduced tolerance to exercise. Previous studies have shown that postmenopausal women and OVX treated rats exhibited decreased muscle performance [57, 58]. Therefore, this study suggests that a loss of functional capillaries in skeletal muscle, associated with decreased circulating ovarian hormones, may influence muscle metabolic dysfunction, such as exercise intolerance, in postmenopausal states. Furthermore, OVX in the present study induced exercise intolerance, the abnormal capillarity possibly due to the angiostatic effects of TSP-1 and the increased mitochondrial enzyme activity, which are similarly observed in the skeletal muscle of peripheral arterial disease. Hence, the present study demonstrated for the first time that OVX could induce peripheral arterial disease-like alteration in the capillarity and mitochondrial activity of skeletal muscle.

Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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