Development of Electrical Stimulation System of Skeletal Muscle to Improve Walking Performance in Rat Claudication Model

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Momoko OGITANI

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Momoko OGITANI

Table of Contents

General Abstract

Intermittent claudication (IC) is the most common symptom of peripheral arterial disease (PAD) caused by narrowing in the main artery. Claudication is pain caused by little blood flow to muscles during physical activities such as walking. Patients with IC fell pain in lower limbs and gets better after resting for a few minutes, thus IC significantly deteriorates quality of life of patients. There is no clinical agent which can improve walking ability in patients with IC, therefore, searching new therapeutic targets for treatment of IC is imperative. For treatment of IC, exercise training is the most effective; however, the underlying mechanisms to improve walking distance remain elusive. Because skeletal muscle is a center part where exercise training converts mechanical loading into molecular signals such as improvement of microcirculation, energy metabolism, and cytokine secretion, the function of skeletal muscle could largely contribute to amelioration of walking ability. In this research, I compared rat IC models and selected the best one that mimicked human IC pathology. Furthermore, I confirmed this IC model was suitable for analysis of the mechanism by which walking ability improved.

For the purpose of elucidation of the local mechanisms by which exercise training improves walking performance in claudicants, I focused on an analysis of skeletal muscle in animal IC model. To mimic the mechanical loading in skeletal muscle by exercise training, I developed an implantable device to locally induce ischemic skeletal muscle contraction mimicking exercise training via electrical stimulation (ES). Rats were assigned to four groups, Sham, Ischemia (Isch), Isch + exersice (EX) and Isch +

ES groups. EX and chronic low-frequency ES of ischemic skeletal muscles significantly recovered the occlusion-induced walking impairment in the model. Furthermore, both EX and ES significantly increased capillaries in the ischemic skeletal muscles and shifted the muscle fibers toward oxidative types. These findings demonstrate that ES takes on common features of exercise in the rat claudication model, which may facilitate investigations on the local mechanisms of exercise-induced functional recovery.

To analyze transcript profiling, I confirmed gene expression following hind-limb ischemia, and recovery following EX or ES-intervention using GeneChip® microarray. Of the top 40 genes upregulated or downregulated following EX and ES compared with Isch, most genes changed in opposite directions to sham-operated normal control. This result indicates that EX and ES could recover the changes in gene expression by hind-limb ischemia. I further confirmed the gene expression changes using semi-quantitative real-time PCR. *Dpp4*, *Nov*, and *Ptges* significantly responded following EX and ES, thus they are candidate genes that might potentially be involved in the improvement of walking performance. Further analyses of gene expression profiles and characterization of individual genes will help in the identification of therapeutic targets for the treatment of patients with IC. This research is extremely important not only for selecting appropriate animal IC model for mechanism analysis also for providing a new insight of possible therapeutic targets.

Abbreviations

PAD, peripheral arterial disease

IC, intermittent claudication

ABI, ankle-brachial index

Isch, ischemia

ES, electrical stimulation

EX, exercise

IAO, iliac artery occlusion

FAO, femoral artery occlusion

PCR, polymerase chain reaction

TA, tibialis anterior

MHC, myosin heavy chain

General Introduction

Peripheral arterial disease (PAD) is a manifestation of systemic atherosclerosis that affects more than 200 million worldwide [1, 2] and confers an increased risk of cardiovascular morbidity and mortality [3]. The arterial narrowing or occlusion that occurs as a result of the atherosclerotic results in a reduction of blood flow to the lower limb during exercise or at rest. The classic symptom of PAD is pain in the legs with physical activity such as walking, which extremely lowers quality of life of patients. It is shown that PAD patients with higher physical activity have reduced mortality and cardiovascular events compared with PAD patients with the lowest physical activity during daily life [4]. Successful implementation of medical therapy aimed at higher physical activity is critical to reduce the cardiovascular morbidity and mortality in patients with PAD.

Intermittent claudication (IC) is the most commonly observed symptom of PAD. IC is caused by narrowing or blockage in the main artery taking blood to femoral artery, which causes cramp and pain in lower limbs and gets better after resting for a few minutes. Several studies that have assessed patients with walking impairment questionnaire and measurement of walking times demonstrated that reduced walking ability is associated with higher rates of cardiovascular events and death [4, 5]. Conversely, it seems to be obvious that improvement in walking distance of patients with IC contributes to improved mortality and quality of life.

Lower extremity revascularization is the preferred management strategies in clinical practice and strongly recommended by current guidelines [6, 7]. Revascularization is a

reasonable treatment option for patients with PAD who have an inadequate response to other guideline-directed therapies. The combination of exercise training and revascularization provides greater benefit than either alone, but further trials with longer follow-up are needed for the outcome of subsequent revascularization [8]. Because of the variability of ischemic limb symptoms, patients should be selected for revascularization on the basis of severity of their symptoms.

In managing PAD, the pharmacotherapy of the highest priority is to modify risk factors that enhances the progression of atherosclerosis and atherosclerotic complications, including lifestyle modification [1]. Any lifestyle change that reduces the risk factors for PAD such as smoking cessation and diet for weight loss has shown to be beneficial, however, they are not enough for improvement of walking ability. In pharmacotherapy, medication to improve circulatory flow such as antiplatelet therapy and statin therapy is considered to be important because pathogenesis of walking impairment in patients with PAD reflects inadequate augmentation of skeletal muscle perfusion during exercise [9]. Cilostazol, a vasodilator with antiplatelet effect, is an effective therapy to improve symptoms and improve walking distance in patients with PAD. However, the usefulness of the drug is limited by its adverse effect profile such as dizziness, gastrointestinal symptoms and its contraindication for use in patients with heart failure. Pentoxifylline, a drug that increases red blood cell deformability does not improve maximal walking distance in patients with PAD, and guidelines do not recommend using it for treatment of claudication. These two are the only drugs approved by Food and Drug Administration (FDA) as a pharmacologic intervention with limited effect [10-12].

Because none of the standard medical therapies directly affects lower limb blood flow, the systemic delivery of angiogenic growth factors have tested over the past decades [13]. The main goal of angiogenic therapy is to promote development of new arterial vessels and improve perfusion of ischemic tissues. Angiogenic growth factors such as vascular endothelial growth factor, fibroblast growth factor, and hepatocyte growth factor, administered intramuscularly or intra-arterially. Furthermore, autologous progenitor cell therapy with bone marrow or adipose-derived progenitor cells administered intra-arterially or intra-muscularly. They promoted angiogenesis and developed collateral vasculature in preclinical studies. However, all of them failed to confirm their efficacy in clinical studies. One of the barriers to success of clinical trials is the difficulty of delivery of angiogenic factors to the right lesion area. For example, after bone marrow-derived mononuclear cells were administered, endothelial cell proliferation occurred in the distal area, not at the site of injection [14]. This result showed that angiogenesis did not occur in the intended lesion and failed to improve perfusion of ischemic tissue sufficiently. Considering the lack of perfusion, it is important to think of a strategy that directly improves the function of ischemic lesion, i.e., ischemic microvessels and skeletal muscle.

Current guidelines endorse supervised exercise therapy as a first-line treatment for all patients with PAD. Clinical studies reported that a supervised exercise program resulted in superior treadmill walking performance compared with stent revascularization [15] and cilostazol treatment [16, 17] in patients with IC. However, exercise training has not been found to improve the ankle-brachial index (ABI), which means the ratio of ankle and brachial (arm) systolic blood pressures and indicates the degree of lower limb ischemia, in patients with IC [15]. This result suggests that exercise training does not

directly treat systemic atherosclerosis. Instead, exercise training may increase the local muscle oxygen supply through microvascular alterations such as formation of new collateral blood vessels and improvement of endothelial vessel dilation [18]. Supervised exercise training improved ability to increase microvascular calf muscle blood flow and oxygen extraction during physical activity [19]. Furthermore, exercise training allowed the muscle to more efficiently use substrates for ATP production and become more resistant to fatigue. In other words, skeletal muscle fibers adapted towards more oxidative [20-22]. Thus, enormous researches have shown that exercise training could directly affect the local ischemic lesion to improve walking performance in patients with IC. Furthermore, exercise training significantly ameliorate the symptoms of various chronic pathologies by reducing inflammation and insulin resistance, improving mood and general well-being, lowering stress levels and even positively influencing cognitive function [23]. These systemic effects have shown to be mediated by signaling molecules produced and secreted by skeletal muscle in response to exercise training [24]. For these reasons, the pleiotropic effect of exercise training focusing on skeletal muscle is an attractive area for basic research as well as for searching a novel therapeutic target that mimics the beneficial effects of exercise training [25].

To reveal the mechanism that exercise training improves the function especially in ischemic skeletal muscle, it is important to analyze the simplified intervention models or clinical samples. When exercise training is implemented, many of systemic adaptations such us increased cardiac output occur and affect skeletal muscle. For eliminating these effects, electrical stimulation (ES) of skeletal muscle is an ideal intervention that could mimic exercise training only in skeletal muscle. Chronic low frequency ES is known to mimic many of the effects of endurance exercise [26]. In

healthy animal skeletal muscle, ES elevates oxidative enzyme capacity and capillary supply, thereby improves fatigue resistance [27]. Also in healthy human studies, ES elevated the levels of oxidative enzymes and increased capillarity [28, 29]. Furthermore, chronic calf muscle ES significantly increased pain-free walking distance and maximum walking distance in patients with IC [26].

Regardless of the fact that exercise training and ES could affect ischemic skeletal muscle and improve walking ability in patients with IC, they are hardly implemented in clinical situation because of less facilities or not covered by insurance. If mechanisms or molecules that exercise training and ES improves the function of ischemic skeletal muscle were revealed, they could be therapeutic targets for treatment of IC. In this study, in order to analyze the mechanisms, first I confirmed that both of exercise training and ES improved walking performance in IC model in rats. Furthermore, I analyzed the expression levels in skeletal muscle of these models and disclosed the potential genes that altered by exercise training and ES in common.

Chapter 1

Neuromuscular stimulation ameliorates ischemia-induced walking impairment in rat claudication model

Abstract

Intermittent claudication (IC) is the most common symptom of peripheral arterial disease which significantly deteriorates quality of life of patients. Exercise training is by far the most effective treatment for IC; however, the underlying mechanisms remain elusive. To determine the local mechanisms by which exercise training improves walking performance in claudicants, I developed an implantable device to locally induce ischemic skeletal muscle contraction mimicking exercise via electrical stimulation (ES). Rats were assigned to four groups, Sham, Ischemia (Isch), Isch + exersice (EX) and Isch + ES groups. Following both unilateral femoral and iliac artery occlusion, rats showed sustained impairment of walking performance in treadmill test. Chronic low-frequency ES of ischemic skeletal muscles for 2 weeks significantly recovered the occlusion-induced walking impairment in the rat claudication model. I further analyzed the ischemic skeletal muscles immunohistochemically following ES or EX; both ES and EX significantly increased capillaries in the ischemic skeletal muscles and shifted the muscle fibers toward oxidative types. These findings demonstrate that ES takes on common features of exercise in the rat claudication model, which may facilitate investigations on the local mechanisms of exercise-induced functional recovery.

Introduction

IC is characterized by fatigue, numbness, cramping, or pain of muscles resulting in decreased walking capability and significant deterioration of quality of life. Despite the large population of patients, the medication for IC is limited; cilostazol and pentoxifylline are the only drugs approved by Food and Drug Administration as a pharmacologic intervention with limited effect [10-12]. Thus, PAD is a disease with high unmet medical need, and novel effective therapeutics are needed.

Many trials have been conducted for decades to find an effective target for patients with PAD mainly focusing on vascular intervention; however, most of the approaches have been unsuccessful [30, 31], implying novel and distinct strategies would be necessary for the treatment of the disease. Although PAD is primarily caused by vascular occlusion, symptoms may arise from multi-dysfunction of peripheral nervous system, skeletal muscles, and vasculature which are functionally interconnected [32]. Therefore, it may be necessary to take an integrative approach targeting the vasculature, nervous system, and skeletal muscles as a whole to tackle the symptoms of PAD.

Exercise is considered as the most efficacious intervention for improving walking capacity in patients with IC, therefore, exercise training is recommended as the first-line treatment of claudication [33]. Exercise is also effective for the management of systemic disease, including improvement of coronary risk factors commonly associated with PAD [34, 35]. Despite its benefits, the underlying mechanisms precipitating this effect remain unclear. Thus, the pleiotropic effect of exercise is an attractive area for basic research as well as for searching a novel therapeutic target that mimics the beneficial effects of exercise [25].

To understand the underlying mechanisms of improvement in walking performance after exercise training, generating a simplified model that reflects a key aspect of exercise is of profound benefit, as exercise exerts multiple effects ranging from local actions on affected limbs to systemic cardiorespiratory function, and even higher brain functions including the learning process [36-39]. I posited that motor nerve-mediated skeletal muscle contraction during exercise is one of the critical aspects that may lead to functional improvement of the affected limb, as exercise is known to induce various adaptations within active skeletal muscles [40-42], even at an older age [43]. Since electrical stimulation (ES) of skeletal muscle could induce intended repetitive muscle contraction, this would be a feasible approach for precipitating the local beneficial effects on the affected limb, while eliminating the systemic effects of exercise.

So far, no report has shown that ES can improve walking performance in experimental animal models of IC. If ES could improve walking distance in experimental animals with IC, this model would be beneficial for analyzing local mechanism by which exercise improves walking performance in skeletal muscle. In this study, in order to analyze the mechanism, first I tested whether ES improves walking distance in rat model of IC using the skeletal muscle stimulator implanted to the affected limb. Chronic stimulation of ischemic skeletal muscle at a low frequency mimicking the endurance exercise, which is known to be beneficial in PAD patients [44], significantly improved the walking performance in the rat IC model. I further demonstrated both ES and exercise training significantly increased the capillary-to-fiber ratio and shifted the muscle fibers toward oxidative types in ischemic muscles.

Materials and Methods

Animals

Eight- to 9-week-old male *F344/DuCrlCrlj* rats were purchased from Charles River Laboratories Japan Inc. (Tokyo, Japan). The animals were housed in a room with 12:12 h light-dark cycle, and had access to water and normal chow diet *ad libitum*. Animals were maintained in an AAALAC-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Institutional Animal Care and Use Committee of Daiichi Sankyo Company, Limited. All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Treadmill test

The walking performance of the rats was examined by treadmill testing procedure. After acclimatization to housing environment and handling, animals were trained to walk on the treadmill apparatus TM-R-N1 (Osaka Microsystems, Osaka, Japan) for 6 days, and gradually acclimated to the speed and duration of the measurement conditions. In the treadmill test, I examined the walking distance of the animals in gradually accelerating conditions at an incline of 15°. The walking speed was initially set at 15 m/min, and increased 5 m/min every 5 min up to 30 m/min. The measurement of walking distance was terminated when the rats stopped walking on the treadmill apparatus and remained near the electrical grid without walking forward, or stayed on the electrical grid for 10 s. The total walking distance was calculated by multiplying the total walking time by the belt speed.

Measurement of hind-limb blood flow

The hind-limb blood flow was analyzed with a Laser Doppler Perfusion Imager PeriScan PIM III (PERIMED, Järfällä, Sweden) under anesthesia with inhalation of 2% isoflurane (Mylan, Pittsburg, PA, USA). The animals were positioned prone on a warming pad set at 37°C. Laser Doppler scans of the plantar surfaces of both hind limbs were performed. The blood flow was expressed in arbitrary units (perfusion units) and reported as the ratio of the Laser Doppler flux of the ischemic (right) leg to that of the non-ischemic (left) leg.

Surgical procedures to induce ischemia

Surgical procedures were performed as previous described [45-47]. The animals were anesthetized through inhalation of 2% isoflurane throughout the surgery. The right iliac artery was occluded by using silk suture approximately 5 mm below the bifurcation from the aorta (iliac artery occlusion [IAO]). Immediately or 2 weeks after IAO, the right femoral artery was occluded below the branching of the arteria profunda femoris (femoral artery occlusion [FAO]). As shown in Fig. 1a, I defined these ischemic models as IAO+4FAO and IAO+2FAO, respectively. I confirmed that the hind-limb blood flow of the right leg had decreased to approximately 20% of that of the left leg after both occlusions. Sham-operated rats were treated in the same manner except occlusion. The animals were allowed to recover for 3 days before the initiation of exercise training or ES. At 4 weeks post-IAO, the animals were euthanized, and muscle samples were collected, weighed, and rapidly frozen using isopentane (2-methylbutane, 26404-75; Nacalai Tesuque, Inc., Kyoto Japan) cooled

with liquid nitrogen.

Exercise training protocol

The animals of the exercise group had run on a treadmill apparatus twice daily for 2 weeks. Each day, the animals exercised both in the morning and afternoon, with at least 4 h between the two exercise bouts. The exercise training condition was 15° incline, 15 m/min for 20 min. As shown in Fig. 1b, exercise was started 3 days after FAO surgery, and implemented 5 days a week for 2 consecutive weeks.

Implantation of electrodes and ES protocol

Under anesthesia with inhalation of 2% isoflurane, each animal of ES group underwent implantation of a custom-designed preprogrammed stimulator (Bio Research Center Co. Ltd., Tokyo, Japan) under the skin of their back at the same time as FAO surgery, connected to electrodes sutured in the vicinity of the right peroneal nerve so as to stimulate the tibialis anterior (TA) muscle. Each set of stimulation was programmed to stimulate at 10 Hz (pulse width: 0.3 ms, intensity: 3 V) for 15 min with a rest period of 85 min, seven sets per day. As shown in Fig. 1b, similar to exercise training, the stimulation was started 3 days after FAO surgery and implemented 5 days a week for 2 consecutive weeks.

Immunofluorescence analysis

Immunohistochemistry techniques were used for capillary density analysis and fiber type determination. Frozen muscle sections (5 μm) were cut in a cryostat (CM3050S; Leica Microsystems, Wetzlar, Germany) on microscope slides. The slides were allowed

to reach room temperature and permeabilized with 0.3% Triton X-100-PBS for 10 min at 4°C. A blocking solution of 5% normal goat serum (NGS)-PBS was applied for 1 h at room temperature, followed by incubation with primary antibodies in 1% NGS-PBS at 4°C overnight. Three consecutive washes with PBS for 5 min each were followed by sequential incubation with secondary antibodies. Primary antibodies against MHCI (BA-F8), MHCIIa (SC-71), and MHCIIb (BF-F3) were purchased from Developmental Studies Hybridoma Bank (University of Iowa, IA, USA) and primary antibody against CD31 was purchased from BD Biosciences (550300; San Jose, CA, USA). Secondary antibodies, Alexa Fluor® 350 Goat anti-mouse IgG_{2b} (A21140), Alexa Fluor® 488 Goat anti-mouse IgG_1 (A21121), and Alexa Fluor® 568 Goat anti-mouse IgG_M (A21043) were purchased from Invitrogen (Carlsbad, CA, USA). Dilution of primary antibodies and secondary antibodies was 1:100 and 1:500 respectively. Images were captured under a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan). After staining, the oxidative core of TA where fiber size is smaller and fibers are more oxidative was analyzed at 20Ⅹ magnification. The detailed illustration of oxidative core was shown in previous reports [48, 49]. The capillary density was determined by counting the total number of capillaries and muscle fibers, and results were expressed as the ratio of capillaries per muscle fiber. Similarly, each percentage of type I, type IIa, type IIb, and type IIx (unstained) fibers was determined. The relative numbers of capillaries and the fiber types were quantified by counting 4 fields per rat. The mean value was calculated by using the average of each rat.

Statistical analysis

Statistical analyses were performed with SAS® System Release 9.2 (SAS Institute Inc., Cary, NC, USA). Data were presented as means ± SE. Comparison analysis between multiple groups was performed using Dunnett's test. A value of $P < 0.05$ was considered significant.

Results

Rat model of chronic hind-limb ischemia

In search for an optimal model for intermittent claudication (IC), I created various hind-limb ischemia models in rats and compared. In order to choose an appropriate IC model, I set two criteria for the selection of the IC model based on the clinical phenotype of IC. First, the model has sustained walking impairment due to limb ischemia. Second, the model has minimal tissue damage in the affected skeletal muscle [50-52]. Hind-limb ischemia models that I tested were iliac artery occlusion (IAO), both iliac artery and femoral artery occlusion (IAO+4FAO), and IAO followed by FAO 2 weeks later (IAO+2FAO) as schematized in Fig. 1a. I evaluated the resting plantar blood flow under anesthesia, walking performance in the treadmill test, and weights of skeletal muscles of ischemic limb 4 weeks after IAO surgery. Plantar blood flow was decreased in all three models as compared with sham operated group (Sham) (Fig. 2a). Walking distance was also significantly shortened in all three models as compared with Sham group (Fig. 2b); among them IAO+2FAO model showed the most severe walking disturbance (##: $P < 0.01$ vs. IAO; $P = 0.08$ vs. IAO+4FAO). Skeletal muscle weights in IAO model did not significantly change as compared with those in Sham group, whereas the weights of tibialis anterior (TA), extensor digitorum longus (EDL), and gastrocnemius (GC) skeletal muscles significantly decreased in IAO+4FAO group. The weight of TA in IAO+2FAO group also decreased, but the overall weight loss was much less as compared with that in IAO+4FAO group (Fig. 2c). In the following studies, we used IAO+2FAO as a model of IC where the walking distance was much shortened with the mild loss of muscle weights.

Walking performance following exercise training and ES in the rat IC model

I next tested whether exercise training also improves the walking ability in my rat IC model. Exercise training was started 3 days after FAO surgery and conducted 5 days per week for 2 consecutive weeks (Fig. 1b). The walking distance increased significantly at 1 week after the onset of exercise ($P < 0.01$ vs. Isch), and recovered to the level comparable to sham group at 2 weeks after the exercise onset (Fig. 3a). By contrast, the resting plantar blood flow under anesthesia was not altered by exercise training (Fig. 3b), as observed in the data reported in human IC patients [53].

I next set out to examine whether chronic skeletal muscle stimulation of the ischemic limb by ES could improve walking performance. ES was started 3 days after FAO surgery and conducted 5 days per week for 2 consecutive weeks to mimic exercise training (Fig. 1b). Chronic ES significantly improved walking performance of IC rats; the walking distance after ES reached approximately twice as long as that of ischemic group (Isch) (Fig. 3a). The resting plantar blood flow was not affected by ES, similar to the result following exercise training (Fig. 3b). The weight of TA in Isch group decreased significantly compared to that in Sham group. However, both exercise training and ES did not affect the weight of TA. Collectively, both exercise training and ES improved walking performance without affecting hind-limb blood flow and muscle weights in the rat IC model.

Capillary-to-fiber ratio following ischemia and effects of exercise training and ES

To investigate the underlying mechanisms of improvement in walking performance by exercise training and ES, I analyzed the capillary-to-fiber ratio in TA muscle, the main skeletal muscle innervated by peroneal nerves where stimulating electrodes were

implanted. As shown in Figs. 4a-4e, the capillary-to-fiber ratio was not affected by hind-limb ischemia as compared with the sham group. However, both exercise training and ES significantly increased the capillary-to-fiber ratio (Figs. 4a-4e).

Fiber type shift following ischemia and effects of exercise training and ES

I further evaluated whether muscle fiber types would change in TA muscle oxidative core following ischemia, and following chronic exercise training and ES. Skeletal muscles are known to have a certain level of plasticity, and fiber type shift can be observed following various interventions to adapt for new environments [54]. Figs. 5a-5h show representative images of immunostaining for different muscle fiber types in each group. The proportion of each fiber type was analyzed by counting the numbers of each type fibers and total fibers (Fig. 5i). Hind-limb ischemia significantly increased the proportion of type IIb fiber and decreased that of type IIx fiber. As compared with the ischemic group, both exercise training and ES significantly increased the proportion of type IIa fiber and decreased that of type IIb fiber. The ratio of type IIx fiber was also decreased in ES-treated group. Collectively, both exercise training and ES shifted the fiber types toward more oxidative fibers in TA oxidative core.

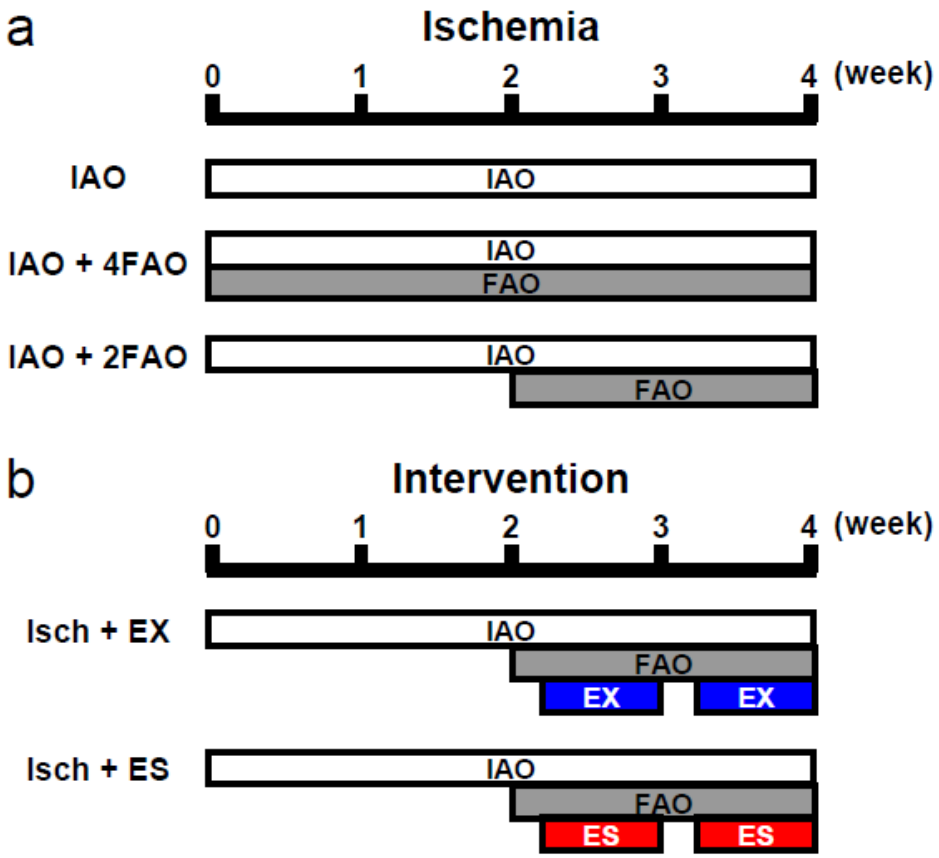


Fig. 1

Schematic diagrams of different ischemic models, exercise training, and ES used in this study. (a) In the iliac artery occlusion (IAO) group, rats were occluded only at the iliac artery and remained occluded for 4 weeks. In IAO+4FAO group, both IAO and femoral artery occlusion (FAO) were simultaneously performed ipsilaterally, and remained occluded for 4 weeks. In IAO+2FAO group, FAO was applied ipsilaterally 2 weeks after unilateral IAO. (b) In IAO+2FAO model (Isch), both exercise training (EX) and electrical stimulation (ES) were started 3 days after FAO and implemented 5 days per week for 2 consecutive weeks

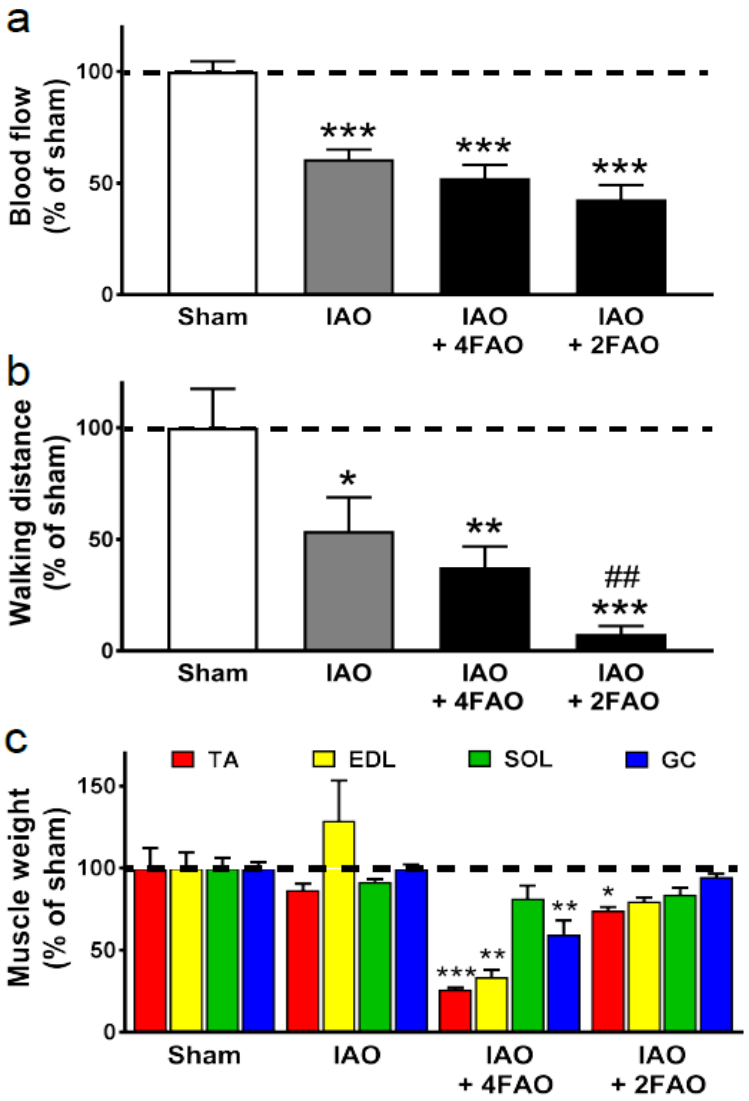


Fig. 2

(a) Plantar blood flow at rest, (b) walking distance, and (c) weight of muscles in each ischemic model (shown in Fig. 1a) at 4 weeks after iliac artery occlusion. Tibialis anterior (TA, red bar); extensor digitorum longus (EDL, yellow bar); soleus (SOL, green bar); gastrocnemius (GC, blue bar). Values are presented as the percentage to mean values of Sham group. n = 5 – 6 rats per group. Values are presented as means ± SE and statistical significance was determined by using Dunnett's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs. Sham group. $^{\#\#}P < 0.01$, vs. IAO group

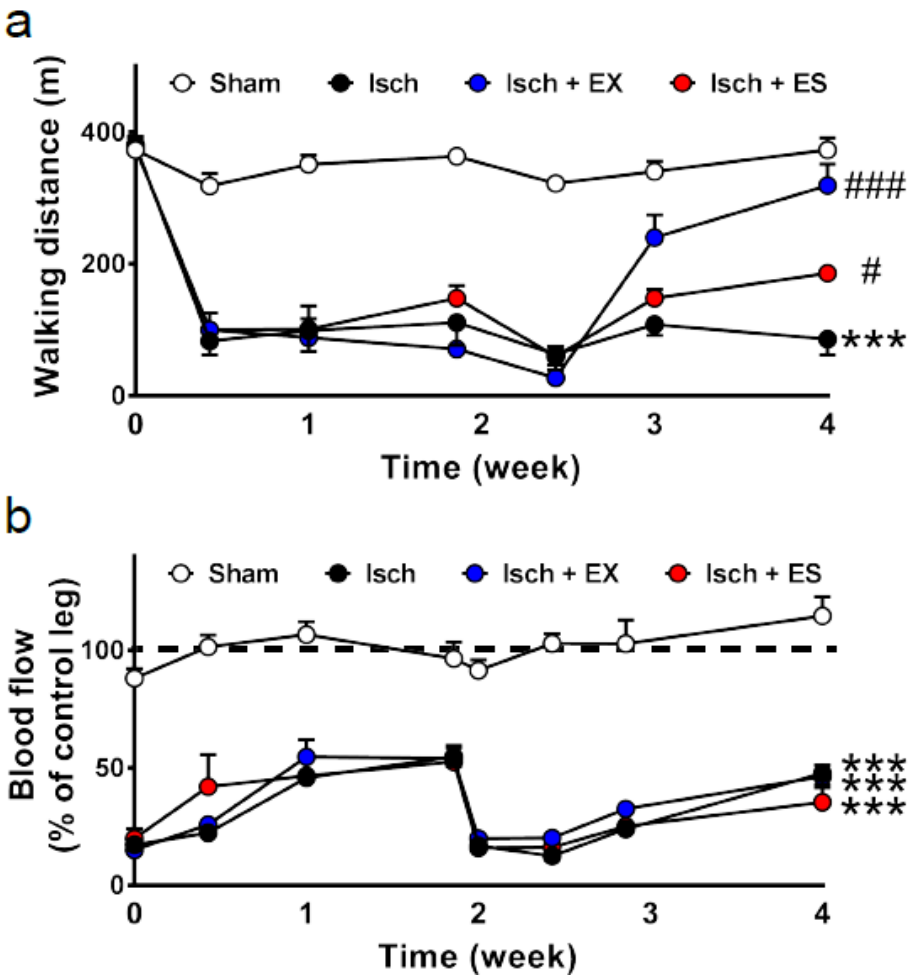


Fig. 3

Effects of exercise (EX) and electrical stimulation (ES) on (a) time-dependent change in walking distance and (b) plantar blood flow at rest in IAO+2FAO ischemic rat model (Isch). The blood flow was expressed as the percentage of the ischemic (right) leg to the non-ischemic (left, control) leg in each rat. White circle, Sham; Black circle, Isch; Blue circle, Isch + EX; Red circle, Isch + ES. n = 5 rats per group. Values are presented as means ± SE and statistical significance was determined by using Dunnett's test at 4 weeks after iliac artery occlusion. ***$P < 0.001$, vs. Sham group. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, vs. Isch group

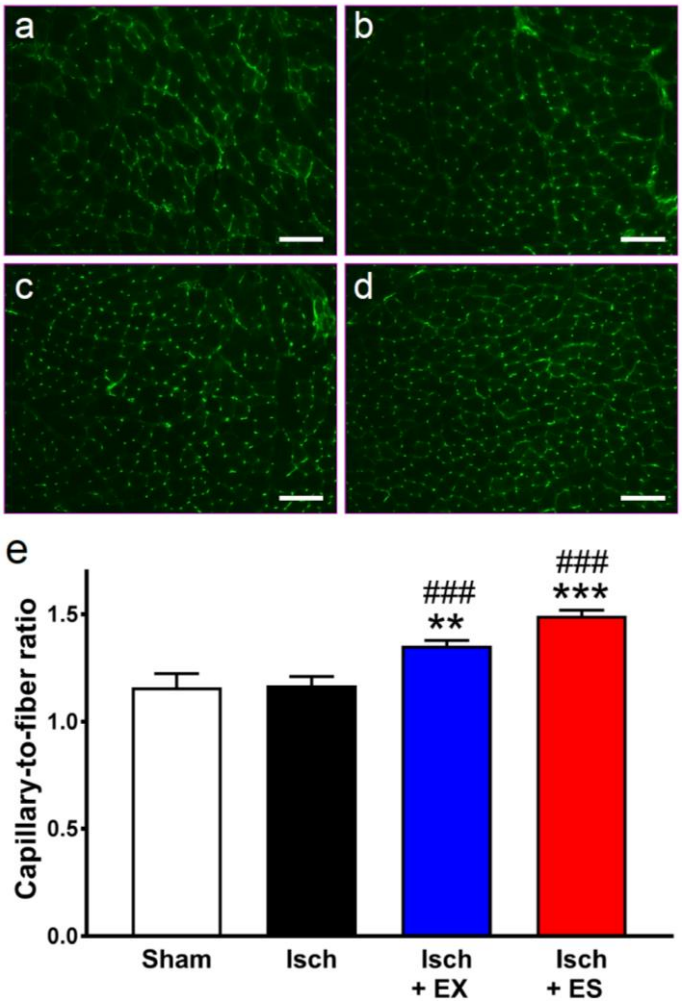
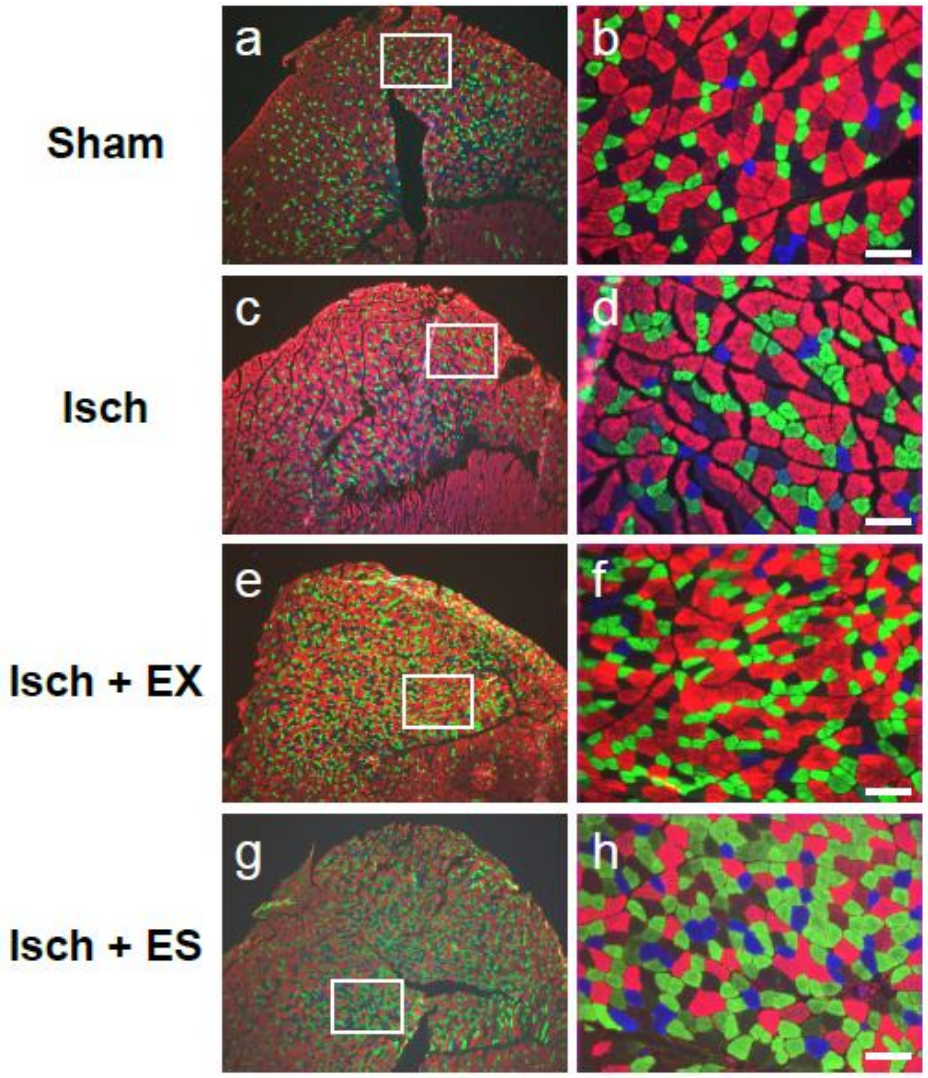


Fig. 4

Capillary-to-fiber ratio of tibialis anterior (TA) oxidative core following ischemia (Isch) and responses to exercise training (EX) and electrical stimulation (ES). (a - d) Representative micrographs of frozen cross sections of CD31 in TA oxidative core from (a) Sham, (b) Isch, (c) Isch + EX, and (d) Isch + ES groups. Scale bar, 100 μm. (e) Capillary-to-fiber ratio in each group. The numbers of capillaries and fibers were counted in 4 fields per rat. n = 5 rats per group. Values are presented as means ± SE and statistical significance was determined by using Dunnett's test. $^{**}P < 0.01$, $^{***}P < 0.001$, vs. Sham group. $^{\#\#\#}P < 0.001$, vs. Isch group


Sham
Isch
Isch + EX
Isch + ES
a
b
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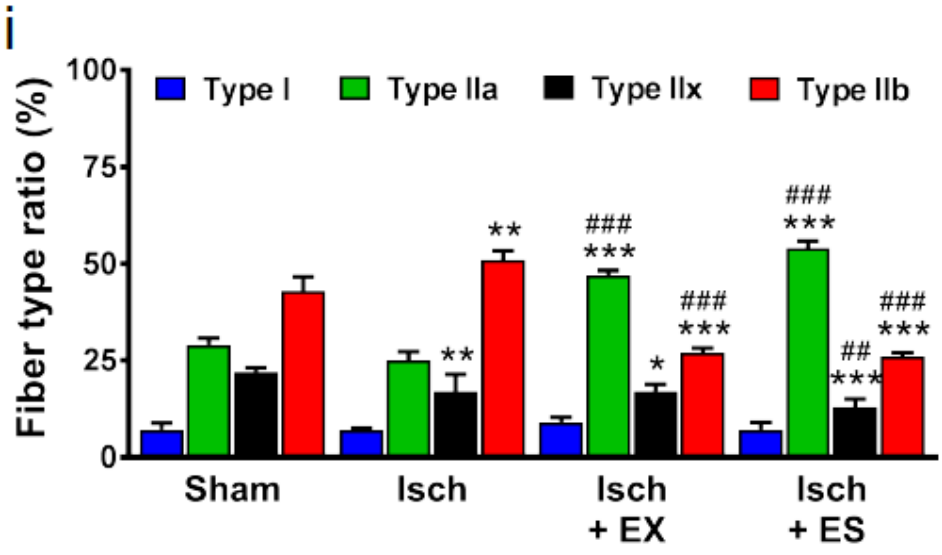
f
g
h


i
Fiber type ratio (%)
100
75
50
25
0
Type I
Type IIa
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Sham
Isch
Isch
+ EX
Isch
+ ES

Fig. 5

Fiber type distribution following hind-limb ischemia (Isch) and effects of exercise training (EX) and electrical stimulation (ES). (a - h) Representative micrographs of frozen cross sections of myosin heavy chain (MHC) isoforms in the tibialis anterior (TA) oxidative core from the Sham (a, b), Isch (c, d), Isch + EX (e, f), and Isch + ES (g, h) groups. Blue, MHC I; Green, MHC IIa; No staining / black, MHC IIx; Red, MHC IIb. Scale bar, 100 μm. (i) Quantification of the fiber type distribution in each group. The fiber type distribution was analyzed as percentage of each fiber type to the total counted fibers. The number of each fiber was counted in 4 fields per rat. n = 5 rats per group. Values are presented as means ± SE, and statistical significance was determined by using Dunnett's test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Sham group. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, vs. Isch group

Discussion

In the present study, I have shown that low-frequency ES to the ischemic limb significantly improved walking performance in the rat IC model. To my knowledge, this is the first study to demonstrate the improvement of walking performance by ES in an experimental model of IC. These results showed a similarity between exercise training and ES in the rat IC model, and suggest that ES could be used as a surrogate intervention for exercise training at least in local aspects. Thus, my established ES model would be advantageous in investigating the local mechanisms of beneficial effects of exercise training focusing on affected limbs.

It is important to use appropriate models that recapitulate the pathophysiology of human diseases. Therefore, I first focused on selecting the appropriate hind-limb ischemic model that mimics human IC phenotype. I set two criteria for the selection of the IC model based on the clinical features of IC. First, the model has walking disturbance due to chronic limb ischemia. Second, the model has minimal tissue damage in the affected skeletal muscle. Following comparison of three different ischemic limb models, IAO+2FAO model was found to be the most appropriate for the IC model which showed sustained walking impairment with minimal tissue damage in the skeletal muscle of the ischemic limb. The 2-week delay of the occlusion of the femoral artery after IAO might have mitigated the severe ischemic damage of skeletal muscle seen in the IAO+4FAO model in which both IAO and FAO were done at the same timing [47]. Moreover, this 2-week delay might have helped establish the chronic ischemic condition by inhibiting collateral vessel development [46]. In addition to these criteria, exercise training, which has been shown to improve the walking performance in patients with IC [15], also improved walking performance in IAO+2FAO rats,

corroborating this model suitable for IC.

By using the selected rat IC model, I investigated the effects of ES on blood flow and walking performance. Remarkably, I found that local ES of ischemic limb significantly improved the walking performance in the rat IC model. Higher-frequency ES is known to induce stronger muscle contraction with quicker muscle fatigue, whereas lower-frequency ES improves fatigue resistance [55]. Consistent with this, I observed that low-frequency ES at 10 Hz was efficacious in improving walking performance. The effect on walking performance of low-frequency ES was smaller as compared to exercise training. Systemic effects of exercise training such as improvement on cardiorespiratory function and the learning effect of walking skills that are absent in ES would explain the difference [56]. Exercise training is known to induce cardiovascular changes and improves endurance performance [57, 58]. Increased cardiac output, the product of heart rate and stroke volume, by exercise training contributes to improved perfusion capacity to muscle permitting for greater oxygen delivery [59]. On the other hand, it is reported that ES does not influence heart rate, blood pressure, and left ventricular hypertrophy [60, 61]. These results may explain the different effect size between exercise training and ES.

The resting blood flow of the ischemic limb did not significantly improve with either exercise training or ES. This is also observed in some clinical reports; the ankle-brachial index, the ratio of blood pressure at the ankle to that of the upper arm at rest which reflects the vascular occlusion of ischemic limb, was not improved following exercise [15, 19, 62-64]. In addition, surgical revascularization does not completely normalize exercise performance. These results suggest that there is a certain mismatch between whole blood flow in the ischemic limb and walking performance. As the pain during

walking in patients with IC is caused by poor blood flow in the affected limb, it might be important to improve the microcirculation during walking [65-67]. Indeed, supervised exercise training was found to increase the maximal calf muscle blood flow during exercise [19]. Measuring limb blood flow during exercise, or evaluating microcirculation in the affected muscle such as TA would be informative in evaluating the beneficial effects of exercise training and ES.

Various potential mechanisms by which exercise training improves walking capacity of patients with IC have been proposed, ranging from systemic effects such as cardiorespiratory function and hemo-rheological effect, to local effects on angiogenesis, endothelial function, muscular function and architecture toward oxidative types, and others [29, 68]. To investigate the potential local mechanisms of exercise training and ES, I first evaluated the capillary-to-fiber ratio in TA muscle which is mainly innervated by peroneal nerve, the site of ES. TA muscle is composed of the glycolytic cortex consisting of glycolytic fibers with larger cross-sectional area (CSA), and the oxidative core which consists of oxidative fibers with smaller CSA including type I fibers. As oxidative fibers are involved in long duration contractile activities and critical for walking performance [69], I analyzed the oxidative core of TA muscle. The capillary-to-fiber ratio in TA oxidative core was not altered by ischemic treatment. However, both exercise training and ES significantly increased the capillary-to-fiber ratio as compared with the ischemia group. The increase in capillary density improves oxygen delivery to the active muscles and increases mitochondrial fatty acid oxidation capacity [20, 70], resulting in skeletal muscle remodeling toward oxidative fiber type [71]. Fiber type shift is also considered as one of the potential mechanisms by which skeletal muscles acquire resistance to fatigue after exercise training [72-74]. I further evaluated fiber

type changes in TA oxidative core following ischemia, and following exercise training and ES. I found that both exercise training and ES increased the proportion of the oxidative type IIa fibers and decreased the glycolytic type IIb fibers. Together with the increased capillary density, the fiber type shift toward oxidative types in TA oxidative core may also have contributed to enhanced oxygen utilization and fatigue resistance resulting in improvement of walking performance in the rat IC model [75]. Despite the similar changes in capillary-to-fiber ratio and the fiber type shift, ES could not improve walking distance to the same extent as exercise training. These results might show that stimulating only the local muscles including TA is not enough to mimic the effect of exercise training.

In summary, I have shown that low-frequency ES in the ischemic limb improved the walking performance in the rat IC model, and mechanistic analyses revealed that ES increased the capillary-to-fiber ratio and induced muscle fiber type shift toward the fatigue-resistant oxidative types, suggesting the improvement of microcirculation and oxygen utilization in the affected limb. The present studies indicated that ES could mimic at least some aspects of exercise training, and that my ES model would be an excellent model to investigate local mechanisms of exercise-induced improvement in walking performance. Further analyses of the beneficial effects of ES may lead to novel therapeutic targets for patients with IC.

Chapter 2

Comparative analyses of gene expression profiles following exercise- and electrical stimulation-induced improvement of walking performance in rat claudication model

Abstract

Exercise is considered to be the most efficacious therapeutic approach for improving walking capacity in patients with intermittent claudication (IC). Despite its enormous benefits, the underlying mechanisms remain unclear. To reveal the local mechanisms underlying exercise (EX) -induced improvement in walking performance, I developed an electrical stimulation (ES) model which exerts local limb muscle contraction that mimics exercise training. This prior study showed ES-induced improvement of walking performance in a rat IC model. In the present study, I confirmed gene expression profiles following hind-limb ischemia, and recovery following exercise or ES-intervention using GeneChip® microarray in a rat IC model. Of the top 40 genes upregulated or downregulated following exercise and ES compared with hind-limb ischemia, most genes changed in opposite directions to sham-operated normal control. I further confirmed the gene expression changes using semi-quantitative real-time PCR, and identified several candidates that might potentially be involved in the improvement of walking performance following exercise or ES including *Dpp4*, *Nov*, and *Ptges.* Further analyses of gene expression profiles and characterization of individual genes will help in the identification of therapeutic targets for the treatment of patients with IC.

Introduction

Intermittent claudication (IC) is the most common symptom of peripheral arterial disease and has a significant impact on the quality of life of patients. IC presents with fatigability, numbness, spasm, or muscle pain resulting in deterioration of walking function due to insufficient blood supply to the affected limbs. Despite the large population of patients worldwide, there are few options for pharmaceutical treatment of IC; to date, cilostazol and pentoxifylline are the only drugs approved by the FDA, and both have limited therapeutic effects [10, 11, 76]. Thus, a novel, effective drug is urgently needed.

Exercise training is considered the most effective therapy for improving the walking ability of patients with IC; it is far more effective than cilostazol treatment [16, 17]. However, supervised exercise training, which is the preferred initial treatment for patients with IC is not feasible for all patients due to its limited provision [77]. Since exercise has potent and pleiotropic effects for the treatment of IC, identification of novel therapeutic approaches that mimic the beneficial effects of exercise is an attractive area of research.

Exercise exerts multiple effects, ranging from local effects on IC-affected limbs to cardiorespiratory effects and even effects on higher brain functions such as the learning process [36-38]. To understand the principal mechanisms underlying improved walking ability after exercise, development of a simplified model that reflects a key aspect of exercise would be of great benefit. As such, I postulated that motor nerve-mediated contraction of skeletal muscles during exercise is one of the key factors that might lead to functional improvement in IC-affected limbs, since exercise is known to induce various adaptations within active skeletal muscles [40-42]. In order to investigate the effects of

skeletal muscle contraction on IC symptoms, in a previous study, I developed an electrical stimulation (ES) system and applied it to a rat IC model [78]. In this earlier study, I generated an IC condition by occluding the iliac artery and femoral artery, and implanted electrical stimulators that induced skeletal muscle contraction of the affected limbs. Both ES and exercise, respectively, significantly improved walking performance in the rat claudication model. Moreover, both ES and exercise increased the capillary-to-fiber ratio and exerted muscle fiber type shifts towards more oxidative types. These results suggested that ES could mimic at least some aspects of exercise, leading to an improvement in walking performance in rats with IC.

Given the overlapping mechanisms of exercise and ES, I postulated that there are also common gene expression patterns in the affected limbs following exercise and ES. In the present study, I comprehensively analyzed gene expression profiles in the skeletal muscles following hind-limb ischemia, and examined the effects of exercise and ES. Intriguingly, of the top 40 genes that were upregulated or downregulated following ischemia, most genes changed in the opposite direction towards sham-operated normal control following exercise and ES. In order to discover new therapeutic approaches for patients with IC, further analyses of common gene expression patterns associated with exercise and ES, and characterization of individual genes were implemented. This could lead to a more developed understanding of the local mechanisms underlying the beneficial effects of exercise

Materials and methods

Animals

Eight- to 9-week-old male F344/DuCrlCrlj rats were purchased from Charles River Laboratories Japan Inc. The animals were housed in a room with 12:12 h light-dark cycle, and had access to water and normal chow diet ad libitum. Animals were maintained in an AAALAC-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Institutional Animal Care and Use Committee of Daiichi Sankyo Company, Limited. All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Surgical procedures of hind-limb ischemia

The surgical procedures for inducing hind-limb ischemia were performed as previously described [78]. Briefly, the animals were anesthetized via inhalation of 2% isoflurane throughout the surgery. The right iliac artery was occluded using a silk suture approximately 5 mm below the bifurcation from the aorta. Two weeks after iliac artery occlusion, the right femoral artery was occluded below the branching of the arteria profunda femoris. Sham-operated rats were treated in the same manner aside from occlusion.

Exercise and ES protocol

The animals in the exercise group ran on a treadmill apparatus (Osaka Microsystems, Osaka, Japan, TM-R-N1) twice daily for two weeks. Each day, the animals exercised both in the morning and afternoon, with at least 4 h between the two exercise sessions. The

exercise training condition was at a 15° incline, 15 m/min for 20 min.

Each animal in the ES group underwent implantation of a custom-designed preprogrammed stimulator (Bio Research Center Co. Ltd., Tokyo, Japan) under the skin of their back at the same time as femoral artery occlusion; the stimulator was connected to electrodes sutured in the vicinity of the right peroneal nerve so as to stimulate the tibialis anterior (TA) muscle. Each set of stimulations was programmed to stimulate at 10 Hz (pulse width: 0.3 ms, intensity: 3 V) for 15 min with a rest period of 85 min; there were seven sets per day. Both exercise and ES were commenced three days after femoral artery occlusion surgery and were implemented five days a week for two consecutive weeks.

On the day after the last day of exercise or ES, the animals were euthanized, and muscle samples were collected.

GeneChip® microarray expression analysis

TA muscle samples were stored at -80°C until RNA extraction. Snap frozen tissue samples were powdered by TissueLyser II (Qiagen, Hilden, Germany, 85300). Total RNA isolation was performed using RNeasy® Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany, 74704) according to the manufacturer's instructions. For gene chip analysis, an equal amount of total RNA from all animals in each group was pooled. Preparation of cDNA, cRNA, hybridization, and scanning of microarrays were performed following the manufacturer's instructions (Affymetrix, CA, USA). To identify differentially expressed transcripts in my analysis the GeneChip® Rat Genome 230 2.0 was employed. The data sets are accessible through the National Center for Biotechnology Information Gene Expression Omnibus database repository (GSE140629).

Real-time reverse transcription-polymerase chain reaction (Real-time RT-PCR)

The extracted RNA was used to synthesize first-strand cDNA with SuperScript VILO Master mix (Life Technologies, CA, USA, 11755-500) and polymerase chain reaction (PCR) was performed with TaqMan® Fast Advanced Master mix (Life Technologies, CA, USA, 4444558) and TaqMan® Custom Arrays (Life Technologies, CA, USA) according to the manufacturer's instruction. The relative amount of the specific mRNA of interest was normalized to reference gene (Rplp, 50S ribosomal protein L16). Analysis of the obtained data was performed using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical analyses were performed with SAS® System Release 9.2 (SAS Institute Inc., Cary, NC, USA). Comparison of animal data and the real-time PCR results between multiple groups were performed using Tukey's test. A value of $P < 0.05$ was considered statistically significant.

Results

DNA microarray analysis

To explore the key genes in the skeletal muscle that are associated with improved walking performance in the IC model, I analyzed gene expression profiles in each group. There were 419 genes in the exercise group and 915 genes in the ES group that were upregulated more than 1.5 times compared with ischemia (Isch) group. On the contrary, 576 genes from Isch + EX group and 853 genes from Isch + ES group were downregulated more than 1.5 times compared with Isch group. Since I aimed to explore the genes that mimic the local effects of exercise, I extracted the genes whose expression were altered in both Isch + EX and Isch + ES groups. As shown in Fig. 6, 81 genes were upregulated (up) and 80 genes were downregulated (down) in both Isch + EX and Isch + ES groups compared with Isch group. Top 40 of upregulated or downregulated genes in Isch + EX and Isch + ES groups are listed in Table 1 and Table 2, respectively, in the order of extent which exercise group varied. Interestingly, 78 of 80 genes changed into the opposite directions of which Isch group changed against sham group.

Real-time PCR analysis

Real-time PCR analysis was performed to confirm the results of the DNA-microarray. Genes shown in Table 2 and Table 3 were analyzed using individual mRNA samples for each group. The analysis revealed that *Dpp4*, *Nov*, and *Ptges* were significantly downregulated in both Isch + EX and Isch + ES groups compared with Isch group (Figs. 7a-7c).

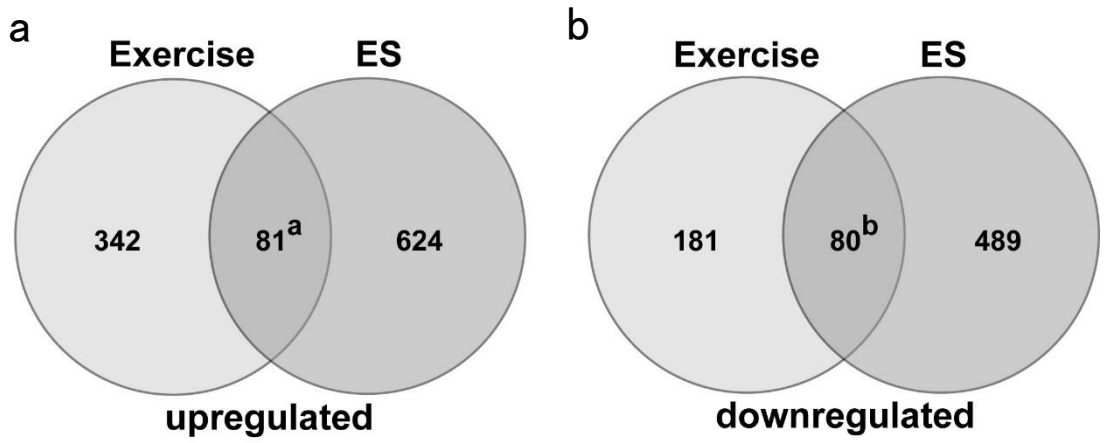

Fig. 6

Venn Diagrams Based on Genes of the DNA Microarray. (a) The number of genes upregulated by exercise or electrical stimulation (ES) compared with ischemia (Isch) [FC (fold change) > 1.5]. (b) The number of genes downregulated by exercise or ES compared with Isch (FC < 1/1.5).

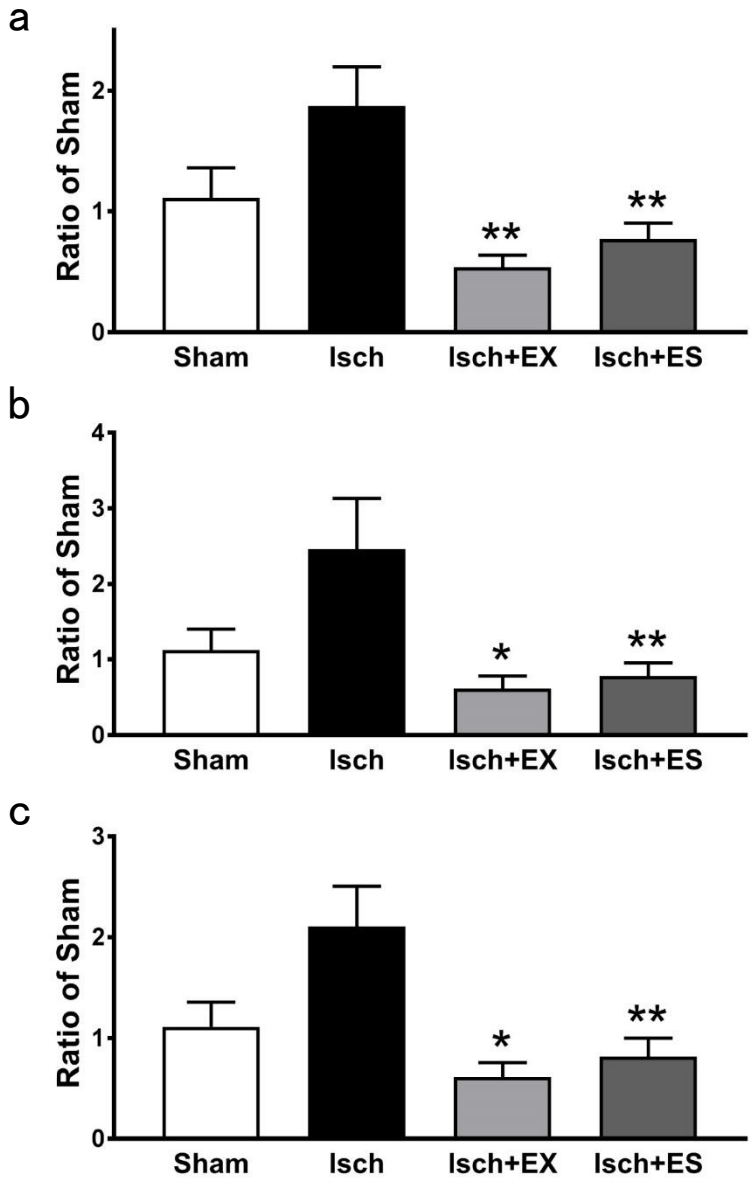

Fig. 7

The relative amounts of Dpp4 (a), Nov (b), and Ptges (c) were normalized to reference gene (Rplp, 50S ribosomal protein L16). Values are presented as ratios of mean values of sham group. Values are presented as mean ± SE and statistical significance was determined using Tukey's test. *P < 0.05, **P < 0.01 vs. ischemia (Isch) group.

Probe Set ID	Gene Name	Gene symbol	Fold change		
			Ischemia vs. Sham	Ischemia+EX vs. Ischemia	Ischemia+ES vs. Ischemia
1393625_at	intersectin 2	Itsn2	0.02 ■	50.66 ■	1.96 ■
1368734_at	cholinergic receptor, nicotinic, delta	Chrnd	0.07 ■	20.7 ■	3.79 ■
1372865_at	zinc finger protein 364	Zfp364	0.1 ■	14.8 ■	1.86 ■
1380236_at	Integrin alpha 9	Itga9	0.11 ■	12.41 ■	1.78 ■
1370165_at	small muscle protein, X-linked	Smpx	0.17 ■	12.05 ■	2.25 ■
1386937_at	ATPase, Na+/K+ transporting, beta 1 polypeptide	Atp1b1	0.11 ■	11.77 ■	2.76 ■
1397867_at	A kinase (PRKA) anchor protein 6	Akap6	0.06 ■	10.95 ■	8.92 ■
1390937_at	similar to chromosome 14 open reading frame 50	RGD1309051	0.11 ■	10.51 ■	1.92 ■
1389265_at	glucan (1,4-alpha-), branching enzyme 1	Gbe1	0.1 ■	9.8 ■	3.31 ■
1389737_at	dystrobrevin alpha	Dtna	0.13 ■	8.05 ■	2.6 ■
1387059_at	serine/threonine kinase 39, STE20/SPS1 homolog (yeast)	Stk39	0.17 ■	7.43 ■	1.96 ■
1390830_at	hypothetical protein LOC499602	LOC499602	0.24 ■	7.13 ■	1.56 ■
1377869_at	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	Ccrn4l	0.19 ■	7.11 ■	3.83 ■
1367660_at	fatty acid binding protein 3, muscle and heart	Fabp3	0.17 ■	6.96 ■	1.66 ■
1379833_at	leucine rich repeat and Ig domain containing 4 /// RAR-related orphan receptor C	Lingo4 /// Rorc	0.18 ■	6.6 ■	1.57 ■
1387036_at	hairy and enhancer of split 1 (Drosophila)	Hes1	0.23 ■	5.93 ■	4.56 ■
1369843_at	cholinergic receptor, nicotinic, alpha 1 (muscle)	Chrna1	0.25 ■	5.86 ■	3.43 ■

1398245_at	synuclein, gamma (breast cancer-specific protein 1)	Sncg	0.25	■	5.7	■	2.44	■
1378745_at	period homolog 3 (Drosophila)	Per3	0.21	■	5.06	■	4.01	■
1392952_at	acyl-CoA synthetase family member 2	Acsf2	0.25	■	4.83	■	1.74	■
1387795_at	polymerase (DNA directed), alpha 2	Pola2	0.49	■	4.78	■	3.81	■
1372595_at	actinin alpha 2	Actn2	0.25	■	4.25	■	3.05	■
1372107_at	four and a half LIM domains 1	Fhl1	0.45	■	3.79	■	1.77	■
1397536_at	similar to KIAA0833 protein	Camta1	0.31	■	3.59	■	1.86	■
1370856_at	actin, alpha, cardiac muscle 1	Actc1	0.33	■	3.55	■	1.79	■
1370390_at	coronin 6	Coro6	0.92		3.52	■	4.23	■
1369590_a_at	DNA-damage inducible transcript 3	Ddit3	0.38	■	3.45	■	2.26	■
1381798_at	LIM domain 7	Lmo7	0.24	■	3.08	■	1.6	■
1387874_at	D site of albumin promoter (albumin D-box) binding protein	Dbp	0.7		3.04	■	2.42	■
1367576_at	glutathione peroxidase 1	Gpx1	0.37	■	2.91	■	1.92	■
1370026_at	crystallin, alpha B	Cryab	0.35	■	2.82	■	1.58	■
1398664_at	GRAM domain containing 3	Gramd3	0.29	■	2.76	■	2.46	■
1388802_at	brain expressed gene 1	Bex1	0.29	■	2.73	■	1.79	■
1375941_at	BAI1-associated protein 2-like 1	Baiap2l1	0.33	■	2.62	■	2.69	■
1372091_at	MID1 interacting protein 1 (gastrulation specific G12 homolog (zebrafish))	Mid1ip1	0.49	■	2.62	■	1.8	■
1389622_at	solute carrier family 25, member 13 (citrin)	Slc25a13	0.49	■	2.53	■	2.28	■
1391030_at	similar to KIAA0833 protein	Camta1	0.41	■	2.52	■	2.31	■
1386965_at	lipoprotein lipase	Lpl	0.44	■	2.49	■	1.52	■

1370157_at	phospholamban	Pln	0.61	■	2.46	■	1.53	■
1372626_at	tumor protein D52-like 1	Tpd52l1	0.63	■	2.4	■	1.94	■

Table 1

The top 40 genes with altered expression from the Isch + EX and Isch + ES groups are listed in the order of the extent of upregulation in the Isch + EX group. The genes downregulated more than 1.5 times in Isch group compared with Sham group are shown in the red column. The genes upregulated more than 1.5 times in Isch + EX and Isch + ES group compared with Isch group are shown in the green column.

Probe Set ID	Gene Name	Gene symbol	Fold change		
			Ischemia vs. Sham	Ischemia+EX vs. Ischemia	Ischemia+ES vs. Ischemia
1390119_at	secreted frizzled-related protein 2	Sfrp2	7.43 ■	0.09 ■	0.3 ■
1396614_at	secreted frizzled-related protein 2	Sfrp2	4.05 ■	0.23 ■	0.37 ■
1377086_at	C1q and tumor necrosis factor related protein 3	C1qtnf3	3.87 ■	0.27 ■	0.62 ■
1388116_at	collagen, type I, alpha 1	Col1a1	3.26 ■	0.29 ■	0.58 ■
1373401_at	Tenascin C	Tnc	2.71 ■	0.33 ■	0.47 ■
1368172_a_at	lysyl oxidase	Lox	3 ■	0.34 ■	0.63 ■
1384063_at	collagen triple helix repeat containing 1	Cthrc1	3.3 ■	0.34 ■	0.6 ■
1378586_at	cytokine inducible SH2-containing protein	Cish	1.19	0.35 ■	0.3 ■
1368394_at	secreted frizzled-related protein 4	Sfrp4	3.24 ■	0.36 ■	0.32 ■
1368171_at	lysyl oxidase	Lox	3.42 ■	0.37 ■	0.59 ■
1368014_at	prostaglandin E synthase	Ptges	1.88 ■	0.41 ■	0.5 ■
1371815_at	microfibrillar-associated protein 2	Mfap2	2.35 ■	0.43 ■	0.67 ■
1371232_a_at	versican	Vcan	1.94 ■	0.43 ■	0.64 ■
1398350_at	hypothetical protein LOC100364588	LOC100364588	2.32 ■	0.43 ■	0.66 ■
1387947_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	Mafb	1.55 ■	0.43 ■	0.66 ■
1370382_at	RT1 class II, locus Db1	RT1-Db1	2.18 ■	0.44 ■	0.56 ■
1393129_at	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide III	P4ha3	2.67 ■	0.46 ■	0.4 ■
1388792_at	growth arrest and DNA-damage-inducible, gamma	Gadd45g	0.93	0.47 ■	0.53 ■

1384437_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1	Smarca1	1.86	■	0.47	■	0.63	■
1383708_at	integrin, beta-like 1	Itgbl1	2.25	■	0.48	■	0.54	■
1381572_at	similar to RIKEN cDNA 1810065E05	RGD1565787	1.69	■	0.48	■	0.65	■
1370042_at	stathmin-like 2	Stmn2	1.89	■	0.48	■	0.54	■
1370892_at	complement component 4, gene 2 /// complement component 4B (Chido blood group)	C4-2 /// C4b	2.51	■	0.49	■	0.59	■
1374616_at	platelet-derived growth factor receptor-like	Pdgfrl	1.93	■	0.49	■	0.56	■
1388265_x_at	versican	Vcan	1.42		0.49	■	0.52	■
1370869_at	branched chain aminotransferase 1, cytosolic	Bcat1	2.71	■	0.49	■	0.55	■
1373148_at	carboxypeptidase X (M14 family), member 2	Cpxm2	1.96	■	0.51	■	0.35	■
1372646_at	similar to RIKEN cDNA 1500015O10	RGD1305645	1.88	■	0.51	■	0.47	■
1368490_at	CD14 molecule	Cd14	1.81	■	0.51	■	0.65	■
1370384_a_at	prolactin receptor	Prlr	1.84	■	0.51	■	0.57	■
1368813_at	CCAAT/enhancer binding protein (C/EBP), delta	Cebpd	1.29		0.51	■	0.52	■
1371754_at	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25	Slc25a25	0.69		0.52	■	0.4	■
1368163_at	dipeptidylpeptidase 4	Dpp4	1.36		0.52	■	0.4	■
1392265_s_at	matrix metallopeptidase 23	Mmp23	1.96	■	0.52	■	0.57	■
1368237_at	tenomodulin	Tnmd	2.5	■	0.52	■	0.36	■
1369152_at	protein phosphatase 3, regulatory subunit B, alpha isoform	Ppp3r1	1.32		0.53	■	0.4	■
1368883_at	nephroblastoma overexpressed gene	Nov	1.64	■	0.54	■	0.55	■

1388233_at	cytokine inducible SH2-containing protein	Cish	1.23		0.54	■	0.38	■
1369484_at	WNT1 inducible signaling pathway protein 2	Wisp2	1.86	■	0.54	■	0.62	■
1389999_at	Eukaryotic elongation factor-2 kinase	Eef2k	1.54	■	0.54	■	0.5	■

Table 2

The top 40 genes with altered expression from Isch + EX and Isch + ES groups are listed in the order of the extent of downregulation in Isch + EX group. The genes upregulated more than 1.5 times in Isch group compared with Sham group are shown in the green column. The genes downregulated more than 1.5 times in Isch + EX and Isch + ES group compared with Isch group are shown in the red column

Discussion

In the present study, I have shown changes in a number of genes in skeletal muscles as a result of exercise and ES interventions, respectively. Among them, *Dpp4*, *Nov*, and *Ptges* were significantly decreased by both exercise and ES following hind-limb ischemia. To my knowledge, this is the first study that comparatively analyzed gene expression profiles following exercise- and ES-induced improvement in walking performance in a rat IC model. My ES model is particularly useful for investigating the local mechanisms underlying the beneficial effects of exercise in the IC affected limbs. The genes identified in the current study might have contributed to the improvement in walking performance via effects on local limbs following exercise. Further analyses are underway to identify the connection between the gene expression changes and the functional outputs of the skeletal muscles.

To explore the genes that were locally affected in the ischemic limbs following exercise, I also extracted the genes that changed following ES. Intriguingly, when I examined the genes that were upregulated in both Isch + EX and Isch + ES groups compared with Isch group, I found they were downregulated by the ischemic condition, and vice versa (with the exception of some genes). These results suggest that skeletal muscle contraction itself might have the potential to restore the damage following ischemia. These changes could have contributed in the improvement in walking performance. The following three genes are especially worthy of discussion.

Dpp4

Dipeptidyl peptidase 4 (Dpp4) is found in many tissues and cells, and known to impair insulin signaling in fat and skeletal and smooth muscle cells [79]. In addition to its insulin

regulation role, Dpp4 is also implicated in angiogenesis. Dpp4 silencing and Dpp4 inhibitors have been shown to inhibit apoptosis of endothelial cells under hypoxic condition and promoted angiogenesis [80-82]. In my previous study, I observed an increased capillary-to-fiber ratio in skeletal muscles with both exercise and ES, respectively. In relation to this finding, decreased expression of Dpp4 might be involved in the increase in microvessels, thus contributing to improved walking ability. Although it has reported that Dpp4 inhibitors are associated with a lower risk of peripheral arterial disease in patients with type 2 diabetes mellitus [83], to date, there is no study investigating whether Dpp4 inhibitors directly affect walking ability in patients with IC or in an animal model of IC. Therefore, future research should investigate the impact of Dpp4 inhibitors on walking ability in IC through the view of both insulin regulation and angiogenesis.

Nov

Nephroblastoma overexpressed gene (NOV) is a member of the CCN gene family and interacts with multiple cell surface receptors including Notch and integrins [84, 85]. With targeted disruption of the Nov gene, it was shown that NOV is a regulator of skeletal muscle and cardiac development, and is is implicated in various disease processes including cardiomyopathy and muscle atrophy [86]. Because NOV is upregulated upon hypoxic treatment, accompanied by stabilization of hypoxia-inducible factor-1α in extravillous trophoblast [87], IC might also upregulated NOV expression in skeletal muscle. In myogenic cells, elevated expression of NOV led to downregulation of MyoD and myogenin, resulting in inhibition of myotube formation [84, 88]. In my study, both exercise and ES downregulated NOV expression. The results suggest that both the ES and

exercise interventions may have promoted myogenic differentiation and mitigated skeletal muscle injury, leading to improvement in walking distance. On the other hand, it has also been reported that NOV is an angiogenic factor and supports wound healing [85, 89]. This does not align with my results that showed that exercise and ES produced an increase in microvessels. Cell type-specific gene expression analyses might provide a better understanding and resolve these inconsistencies.

Ptges (mPGES-1)

Microsomal prostaglandin E synthase- 1 (mPGES-1) is an enzyme responsible for the conversion of prostaglandin H_2, produced by cyclooxygenase (COX) enzymes, into the biologically active PGE_2. mPGES-1 expression is induced under proinflammatory conditions similar to COX-2 expression [90]. Gene deletion studies have confirmed the involvement of mPGES-1 in various disease models such as collagen-induced arthritis, atherosclerosis, and stroke [91-93]. In a mice stroke model, it was suggested that induction of mPGES-1 together with COX-2 expression is required for post-ischemic PGE_2 production, which causes inflammation and ischemia-induced neuronal death. Recently, mPGES-1 inhibitor have been suggested as potential tools for the treatment of pain and inflammation [94, 95]. Assuming that exercise and ES might have downregulated mPGES-1 expression and suppressed inflammation in the ischemic hind-limb, mPGES-1 inhibitors may have the potential to improve walking distance in the rat IC model. However, PGE_2 and its receptors EP3, and EP4 have various roles other than inflammation, including myogenic differentiation and angiogenesis [96-98]. Further investigation on the role of mPGES-1 in ischemic hind-limb is needed.

General discussion

In this study, in order to a analyze the mechanism how exercise training affects ischemic skeletal muscle and improves walking performance, I developed electrical stimulation system in rat IC model and analyzed gene expression profiles with intervention. In the past decades, several angiogenic therapy have been tested to treat patients with PAD [13]. However, none of them did not show any improvement in walking ability and quality of life. These results indicate that development of new main arteries is not enough for treatment and another strategy is needed. In clinical studies, exercise training has shown to be the best intervention that could improve walking performance in patients with IC. Interestingly, exercise training has not been found to improve the ABI, which means exercise training could not promote angiogenesis in main artery or treat arteriosclerosis plaque directly. From this viewpoint, I assumed that the mechanism which exercise training improves walking performance depends on adaptation to ischemia in skeletal muscle, not main artery. To analyze how exercise training affects ischemic skeletal muscle directly, I developed IC model in rats and electrical stimulation system which mimics skeletal muscle contraction of exercise training. Using this ES system, I could analyze the local gene expression profiles in skeletal muscle caused by muscle contraction of exercise training.

First, I developed IC model in rats with ligation of iliac artery and femoral artery. In rodents, surgical ligation of artery induces a number of artifacts as a result of extensive inflammatory injury that can promote angiogenesis [99]. Also in my study, rat model which I ligated only iliac artery or both of iliac artery and femoral artery simultaneously decreased blood flow significantly immediately after ligation, however, blood flow

improved gradually after ligation. On the other hand, rat model which I ligated femoral artery 2 weeks after iliac artery ligation tended to further decrease blood flow than former two models. This result indicates femoral artery ligation inhibited angiogenesis after iliac artery ligation and maintained ischemic state. Consistent with the result of blood flow, the model which was ligated femoral artery 2 weeks after iliac artery showed consistent walking disability compared with other models. This model is important from the point of view that can mimic chronic pathology of patients with PAD. For the sake of searching therapeutic targets, use of appropriate animal model is mandatory, thus I could confirm versatility of this ischemic model.

To simplify the effect of exercise training focusing on skeletal muscle, I applied electrical stimulation system to ischemic skeletal muscle. This system enabled me to cause muscle contraction like exercise training without increase of cardiac output [60]. Because low frequency ES in clinical showed significant improvement of walking performance in patients with IC [44], one aspect of exercise training which contracts skeletal muscle is worthy of research. Low frequency ES is known to mimic many effects of endurance exercise [26]. In this study, I applied low frequency ES to IC model in rats and confirmed that ES improved walking ability. This is the first report showing that ES can improve walking performance in animal IC model. Furthermore, in skeletal muscle of exercise training and ES groups, capillary density increased and muscle fiber type shifted toward more oxidative. These result suggest that ES enabled muscles to utilize oxygen more efficiently, and more effective metabolism led to improvement of walking performance. Because impairment of oxygen uptake contributes to walking intolerance in patients with IC [100], there is the possibility that effective oxygen consumption enables to improve walking ability.

In analyzing gene expression profile in exercise training group, skeletal muscle could be influenced not only by muscle contraction, but also by several hormones during exercise training [101]. In the former part of this study (Chapter1), I revealed ES improves walking distance even without systemic released hormones seen in exercise training. From this result, I assumed that muscle contraction caused by ES evokes enough change of gene expression which contributes to walking tolerance. By extracting genes which changed in exercise training and ES groups commonly, more significant genes supposed to be analyzed. As expected, many genes unraveled to change similarly in exercise training and ES groups using GeneChip® analysis. Furthermore, *Dpp4*, *Nov*, *Ptges* showed reproducibility in semi-quantitative real-time PCR analysis. Dpp4 is most interesting gene in this study because it has already shown to be relevant to PAD [83]. In this previous report, Dpp4 inhibitors lowered extremity amputation risk of patients with PAD, which indicates inhibiting Dpp4 could hamper disease progression. Although Dpp4 is known as a protease which degrades incretin hormones such as glucagon-like peptide 1 (GLP-1) and inhibits insulin secretion, a variety of other effects of Dpp4 have been reported. Dpp4 silencing and Dpp4 inhibitors have been shown to inhibit apoptosis of endothelial cells under hypoxic condition and promoted angiogenesis [80-82]. Considering this role of Dpp4, the change of gene expression in this study occurred in endothelial cells, not in skeletal muscle cells. Because endothelial cells and skeletal muscle cells are related intimately to each other, the change of gene expression in endothelial cells could influenced a property of skeletal muscle cells. Indeed, Dpp4 inhibitor have reported that enhances the energy metabolism in the skeletal muscle of diabetes patients [102] and GLP-1 analogue improved mitochondrial oxygenation activity which led to more exercise capacity in heart failure mouse skeletal muscle [103].

In summary, I showed exercise training and electrical stimulation system improved walking distance in rat IC model and analyzed gene expression profiles which changed in both of the groups. Finally, Dpp4 was emerged as one of the potential targets which could contribute to exercise tolerance. Although Dpp4 inhibitors are used for treatment of diabetes, there is no study investigating whether they directly affect walking ability in patients with IC or in an animal model of IC. Therefore, future research should investigate the impact of Dpp4 inhibitors on walking ability in IC.

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