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ORIGINAL PAPER

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

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Abstract

Intermittent claudication (IC) is the most common symptom of peripheral arterial disease which significantly deteriorates the 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, we 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 + exercise and Isch + ES groups. Following both unilateral femoral and iliac artery occlusion, rats showed sustained impairment of walking performance in the 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. We further analyzed the ischemic skeletal muscles immunohistochemically following ES or exercise training; both ES and exercise training 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.

Keywords Intermittent claudication · Exercise · Electrical stimulation · Skeletal muscle · Mechanism

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]. Intermittent claudication (IC) is the most commonly observed symptom of PAD, which 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 [4–6]. 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 [7, 8], 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 [9]. 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, and is much more effective than cilostazol treatment [10, 11].

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Therefore, exercise training is recommended as the first-line treatment of claudication [12]. A clinical study reported that a supervised exercise program resulted in superior treadmill walking performance compared with stent revascularization in patients with IC [13]. Exercise is also effective for the management of systemic disease, including improvement of coronary risk factors commonly associated with PAD [14, 15]. 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 [16].

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 [17–20]. We 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 [21–23], even at an older age [24]. 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, we 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 [25], significantly improved the walking performance in the rat IC model. We 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 Co., Ltd. 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, we 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 previously described [26–28]. The animals were anesthetized through inhalation of 2% isoflurane throughout the surgery. The right iliac artery was occluded 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, we defined these ischemic models as IAO + 4FAO and IAO + 2FAO, respectively. We confirmed that the hind-limb blood flow of the right leg had decreased

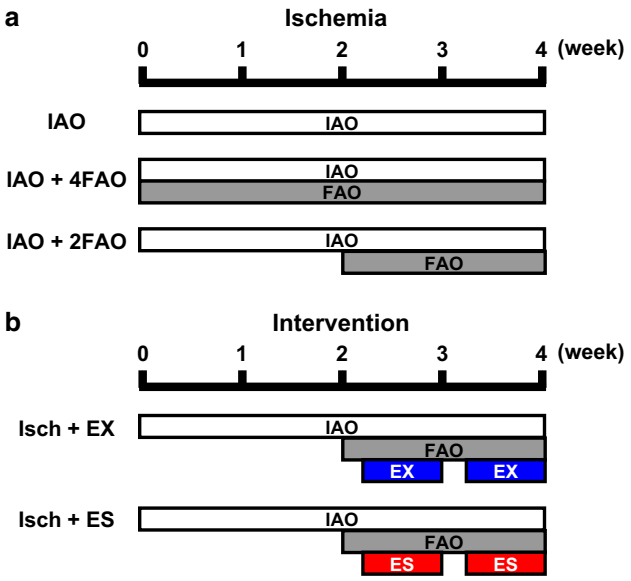

Fig. 1 Schematic diagrams of different ischemic models, exercise training, and ES used in this study. **a** In the iliac artery occlusion (IAO) group, rats were occluded only at the iliac artery and remained occluded for 4 weeks. In IAO + 4FAO group, both IAO and femoral artery occlusion (FAO) were simultaneously 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

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, AlexaFluor 350 Goat anti-mouse IgG2b (A21140), AlexaFluor 488 Goat anti-mouse IgG1 (A21121), and AlexaFluor 568 Goat anti-mouse IgM (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 [29, 30]. 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 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. The results of

statistical analysis of each figures are shown in supplemental tables.

Results

Rat model of chronic hind-limb ischemia

In search for an optimal model for intermittent claudication (IC), we created various hind-limb ischemia models in rats and compared. To choose an appropriate IC model, we 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 [31–33]. Hind-limb ischemia models that we 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. We 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 the 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 the 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 (Supplementary Tables 1, 2, 3).

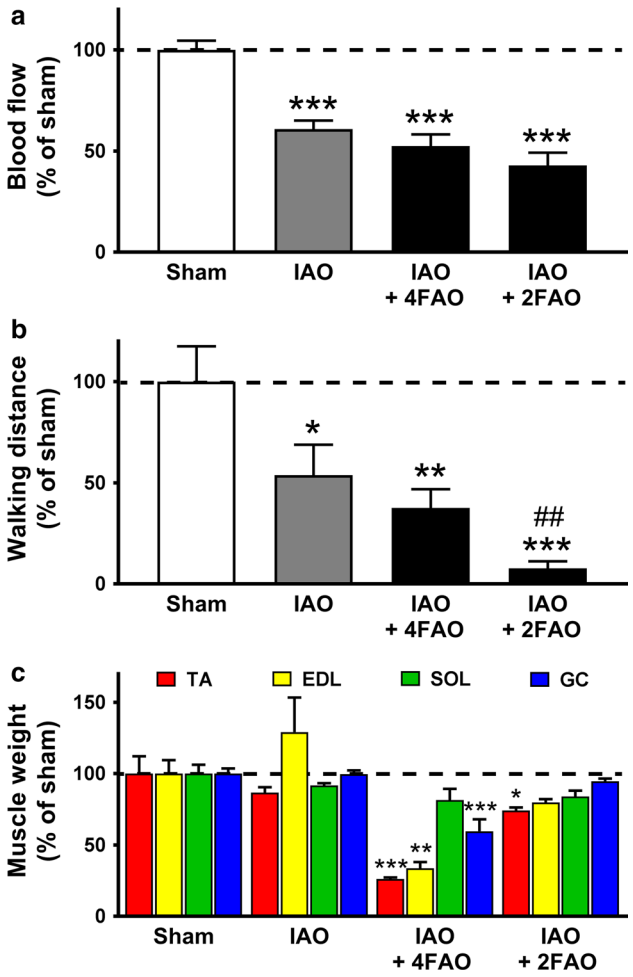

Fig. 2 **a** Plantar blood flow at rest, **b** walking distance, and **c** weight of muscles in each ischemic model (shown in Fig. 1a) at 4 weeks after iliac artery occlusion. Tibialis anterior (TA, red bar); extensor digitorum longus (EDL, yellow bar); soleus (SOL, green bar); gastrocnemius (GC, blue bar). Values are presented as the percentage to mean values of Sham group. $n = 5$–6 rats per group. Values are presented as mean ± SE and statistical significance was determined using Dunnett's test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. Sham group. $^{\#\#}P < 0.01$, vs. IAO group

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

We next tested whether exercise training also improves the walking ability in our 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 [34].

We 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 the ischemic group (Isch) (Fig. 3a). The resting plantar blood flow was not affected by ES, similar to the result following exercise training

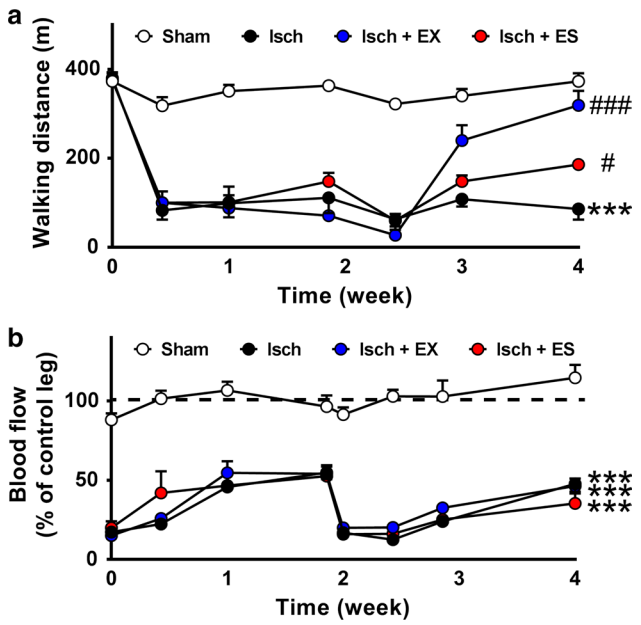

Fig. 3 Effects of exercise (EX) and electrical stimulation (ES) on **a** time-dependent change in walking distance and **b** plantar blood flow at rest in IAO + 2FAO ischemic rat model (Isch). The blood flow was expressed as the percentage of the ischemic (right) leg to the non-ischemic (left, control) 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 mean ± SE and statistical significance was determined 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

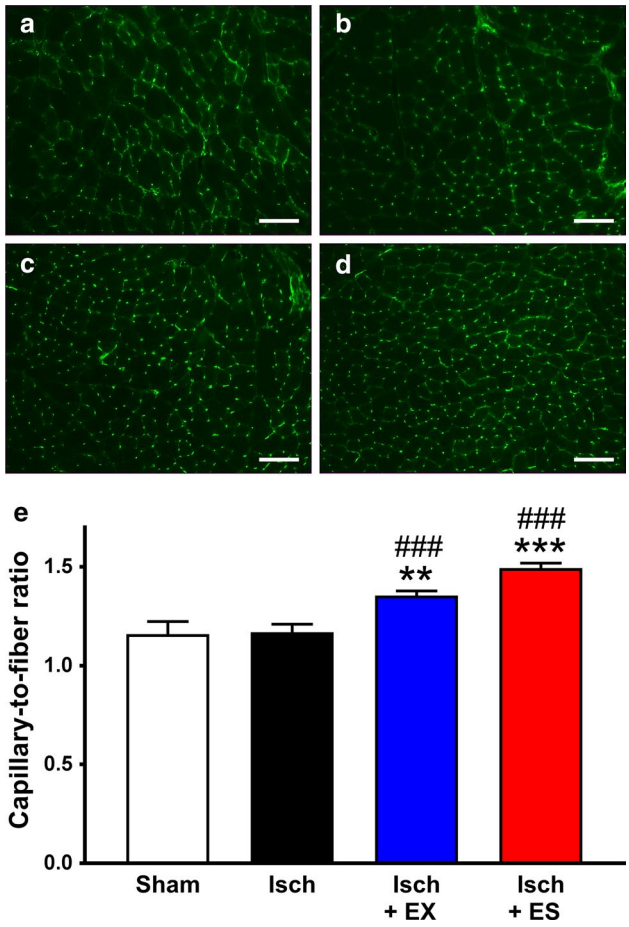

Fig. 4 Capillary-to-fiber ratio of tibialis anterior (TA) oxidative core following ischemia (Isch) and responses to exercise training (EX) and electrical stimulation (ES). **a**–**d** Representative micrographs of frozen cross sections of CD31 in TA oxidative core from **a** Sham, **b** Isch, **c** Isch + 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 mean ± SE and statistical significance was determined using Dunnett's test. $^{**}P < 0.01$, $^{***}P < 0.001$, vs. Sham group. $^{\#\#\#}P < 0.001$, vs. Isch group

(Fig. 3b). The weight of TA in Isch group decreased significantly compared to that in the Sham group. However, both exercise training and ES did not affect the weight of TA (supplemental figure). Collectively, both exercise training and ES improved walking performance without affecting hind-limb blood flow and muscle weights in the rat IC model (Supplementary Tables 4, 5).

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, we 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 Fig. 4, 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 (Fig. 4) (Supplementary Table 6).

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

We 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 [35]. Figure 5a–h shows 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

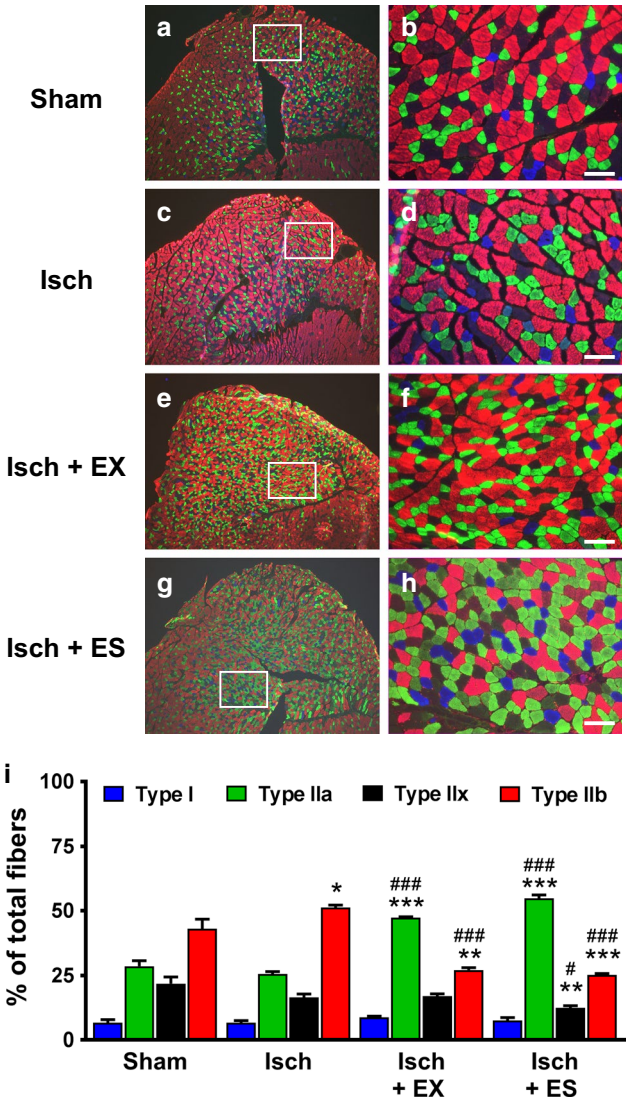

Fig. 5 Fiber type distribution following hind-limb ischemia (Isch) and effects of exercise training (EX) and electrical stimulation (ES). **a**–**h** Representative micrographs of frozen cross sections of myosin heavy chain (MHC) isoforms in the tibialis anterior (TA) oxidative core from the Sham (**a**, **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 the 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 mean ± SE, and statistical significance was determined 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

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 the TA oxidative core (Supplementary Table 7).

Discussion

In the present study, we have shown that low-frequency ES to the ischemic limb significantly improved walking performance in the rat IC model. To our knowledge, this is the first study to demonstrate the improvement of walking performance by ES in an experimental model of IC. Our 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, our 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, we first focused on selecting the appropriate hind-limb ischemic model that mimics human IC phenotype. We 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 [28]. Moreover, this 2-week delay might have helped establish the chronic ischemic condition by inhibiting collateral vessel development [27]. In addition to these criteria, exercise training, which has been shown to improve the walking performance in patients with IC [13], also improved walking performance in IAO + 2FAO rats, corroborating this model suitable for IC.

Using the selected rat IC model, we investigated the effects of ES on blood flow and walking performance. Remarkably, we 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 [36]. Consistent with this, we 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 [37]. Exercise training is known to induce cardiovascular changes and improves endurance performance [38, 39]. 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 [40]. On the other hand, it is reported that ES does not influence heart rate, blood pressure, and left ventricular hypertrophy [41, 42]. 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 [13, 43–46]. 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 [47–49]. Indeed, supervised exercise training was found to increase the maximal calf muscle blood flow during exercise [44]. 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 the walking capacity of patients with IC have been proposed, ranging from systemic effects such as cardiorespiratory function and hemorheological effect to local effects on angiogenesis, endothelial function, muscular function and architecture toward oxidative types, and others [50, 51]. To investigate the potential local mechanisms of exercise training and ES, we 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 [52], we 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 [53, 54], resulting in skeletal muscle remodeling toward oxidative fiber type [55]. Fiber type shift is also considered as one of the potential mechanisms by which skeletal muscles acquire resistance to fatigue after exercise training [56–58]. We further evaluated fiber type changes in TA oxidative core following ischemia, and following exercise training and ES. We 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 [59]. 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, we 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 our 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.

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Author contributions Momoko Shiragaki-Ogitani: conceptualization, formal analysis, investigation, and writing—original draft. Keita Kono: writing—original draft. Futhoshi Nara: conceptualization and writing—original draft. Atsushi Aoyagi: conceptualization, formal analysis, investigation, and writing—original draft.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

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