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Effects of intermittent hyperbaric exposure on endurance and interval exercise performance in well-trained mice

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Abstract

The study was designed to clarify the mechanisms by which hyperbaric exposure (1.3 atmospheres absolute with 20.9% O_2) improves endurance and interval exercise capacities in highly trained mice. Male mice in the training group were housed in a cage with a wheel activity device for 7 weeks from 5 weeks old. Voluntary running markedly increased maximal endurance capacity by 6.4-fold. Trained mice were then subjected to either endurance treadmill training (20–32.5 m min^{-1}) or sprint interval training (5 s run–10 s rest, 30–42.5 m min^{-1}) with (HypET or HypSIT, respectively) and without (ET or SIT, respectively) 1 h hyperbaric exposure for 4 weeks. Maximal endurance capacity was significantly increased by HypET and HypSIT, and maximal interval capacity was significantly enhanced by HypSIT. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha expression levels were markedly increased after HypET and HypSIT. Activity levels of 3-hydroxyacyl-CoA-dehydrogenase, citrate synthase and phosphofructokinase in the red gastrocnemius muscle were increased more by HypET than by ET. Protein levels of mitochondrial transcription factor A, dynamin-related protein-1 and heat shock protein 70 were increased more by HypET than by ET. The proportion of type I fibres in the soleus muscle was remarkably increased by HypSIT. Capillary-to-fibre ratio values in the white gastrocnemius were increased more by HypSIT than by SIT. These results suggest that hyperbaric exposure has beneficial effects for endurance and interval training to improve exercise capacity in highly trained mice.

KEYWORDS
endurance training, hyperbaric exposure, sprint interval training

1 | INTRODUCTION

A commercial hyperbaric apparatus, which functions at <1.5 atmospheres absolute (ATA) with room air (20.9% O_2), has become popular in the field of sports. However, its effects for improving exercise performance have not yet been elucidated clearly. Previous studies showed that acute hyperbaric exposure at 1.25–1.30 ATA for 1 h upregulated mRNA levels of proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Fujita, Tomioka, Ono, & Deie, 2016; Suzuki, 2017) and peroxisome proliferator-activated receptor alpha (PPARα) in rodent skeletal muscle (Suzuki, 2017). Chronic intermittent hyperbaric exposure with endurance exercise training enhanced endurance capacity via the upregulation of PGC-1α and transcription of mitochondrial transcription factor A (Tfam) protein expression levels in mouse skeletal muscle (Suzuki, 2017). In that study, exercise training was conducted for only 4 weeks using adult (12-week-old) untrained mice. Human athletes are more likely to start their athletic career in their childhood and continue to perform a daily training regimen throughout their career. In order to clarify whether hyperbaric exposure has beneficial effects for these highly trained individuals, experiments need to be performed using highly trained animals. Thus, the present study was conducted to examine whether exercise training with intermittent hyperbaric exposure has additive effects for endurance and interval exercise performance in mice that have been trained from an early age.

PGC-1α plays crucial roles in the regulation of skeletal muscle adaptation in response to exercise training (Vega, Huss, & Kelly, 2000; Wu et al., 1999). After muscle contraction, PGC-1α translocates to mitochondria and forms a complex with Tfam, which is upregulated by PGC-1α (Wu et al., 1999). Therefore, mitochondrial biogenesis could be facilitated through the transcription of mitochondrial genome-encoded genes and mitochondrial DNA replication. Acute exhaustive

exercise was previously reported to increase the content of the PGC-1α-Tfam complex remarkably in the mitochondria of mouse skeletal muscle (Safdar et al., 2011).

PGC-1α also contributes to the changes induced in muscle fibre type by exercise training. Although the muscle-specific deletion of PGC-1α did not affect exercise-induced fibre type transformation (Geng et al., 2010), the overexpression of PGC-1α was shown to increase the proportion of type I fibres in skeletal muscle (Lin et al., 2002; Zhang et al., 2017).

Mitochondrial fusion and fission in skeletal muscle are affected by exercise. Mitofusion (Mfn)-1 protein levels in rat skeletal muscle were reduced in response to acute endurance exercise (Ding et al., 2010). In contrast, the phosphorylation of the mitochondrial fission marker dynamin-related protein (Drp)-1 was increased during acute endurance exercise (Pagano, Py, Bernardi, Candau, & Sanchez, 2014). However, the expression of these marker proteins after chronic exercise training has not yet been demonstrated clearly.

High-intensity interval training that consists of repeated all-out efforts (lasting 20–30 s) has been shown to improve muscle oxidative capacity and endurance performance in physically active students (Tabata et al., 1996). To accomplish this training regimen (i.e. 20–30 s of maximal exercise), a specific device, such as a cycle ergometer and treadmill, or a sufficiently broad outdoor field is needed. Short-duration maximal exercise–rest cycles, for example 5 s–10 s, may be beneficial for athletes who do not have regular access to specific devices and/or who cannot exercise outdoors because of harsh atmospheres, such as air pollution and hot or cold temperatures.

In the present study, experiments were designed to elucidate the effects of endurance training or sprint interval training with hyperbaric exposure on exercise capacity, the expression of proteins involved in mitochondrial biogenesis, metabolic enzyme levels and fibre type composition in well-trained mice.

New Findings

- **What is the central question of this study?**
  Intermittent hyperbaric exposure (1.3 atmospheres absolute with 20.9% O_2) enhances endurance capacity by facilitating oxidative and glycolytic capacities in skeletal muscle. It remains unclear whether this strategy enhances endurance performance in well-trained individuals.
- **What is the main finding and its importance?**
  Hyperbaric exposure with endurance training enhanced oxidative and glycolytic capacities and protein levels of mitochondrial transcription factor A, dynamin-related protein-1 and heat shock protein 70. Hyperbaric exposure with sprint interval training increased the proportion of type I muscle fibres and promoted capillary growth and muscle fibre hypertrophy. These results may lead to a new strategy for enhancing exercise capacity in well-trained mice.

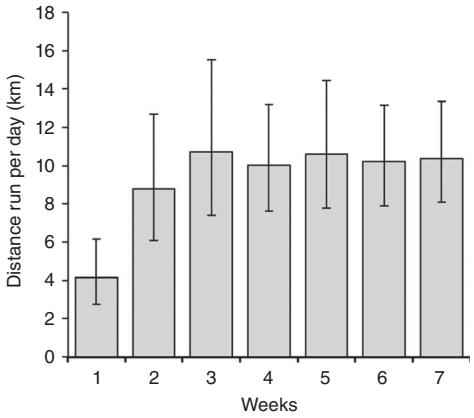

FIGURE 1 Distance run per day during voluntary wheel training. Values are represented as means ± SD

2 | METHODS

2.1 | Ethical approval

All procedures were approved by the Animal Care and Use Committee of Hokkaido University of Education (no. 6, approved on 14 April 2017) and performed in accordance with the 'Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences' of the Physiological Society of Japan and the Editorial by Grundy (2015).

2.2 | Animals and acute hyperbaric exposure

Forty-four male multi cross hybrid mice (4 weeks old) were purchased from Clea Japan Inc. (Tokyo, Japan) and housed in controlled conditions with a temperature of 24 ± 1°C and relative humidity of ~50%. Lighting (07.00–19.00 h) was controlled automatically. All mice were given commercial laboratory chow (solid CE-2; Clea Japan Inc.) and tap water *ad libitum*. After mice had been fed for 1 week and allowed to adapt to the new environment, they were randomly assigned to a sedentary control group (Sed, n = 10) or a training group (n = 34). Mice in the training group were individually housed in a cage with a wheel activity device (13 cm in diameter) for 7 weeks. Wheel activity (distance and running time) was monitored and recorded using digital bike computers (CC-VL820; Cateye Co., Ltd, Osaka, Japan). Mice in the Sed group were housed individually throughout the experiment. To familiarize mice with the treadmill device, all mice, including the Sed group, were subjected to treadmill walking once a week using a controlled treadmill (Modular motor assay; Columbus Instruments Inc., Columbus, OH, USA) for 3 min per day at 10–15 m min^{-1} with a 5 deg incline.

The distance run during voluntary wheel training is shown in Figure 1. After voluntary wheel training, mice were given a 48 h non-exercise period before the maximal endurance capacity test. The test was performed with a graded ramp running protocol using the controlled treadmill as shown in Figure 2a. After the test, mice had a 48 h non-exercise period. The sprint interval capacity test was then performed as shown in Figure 3a, b. Total work (in joules) was calculated as the product of body weight (in kilograms), speed (in

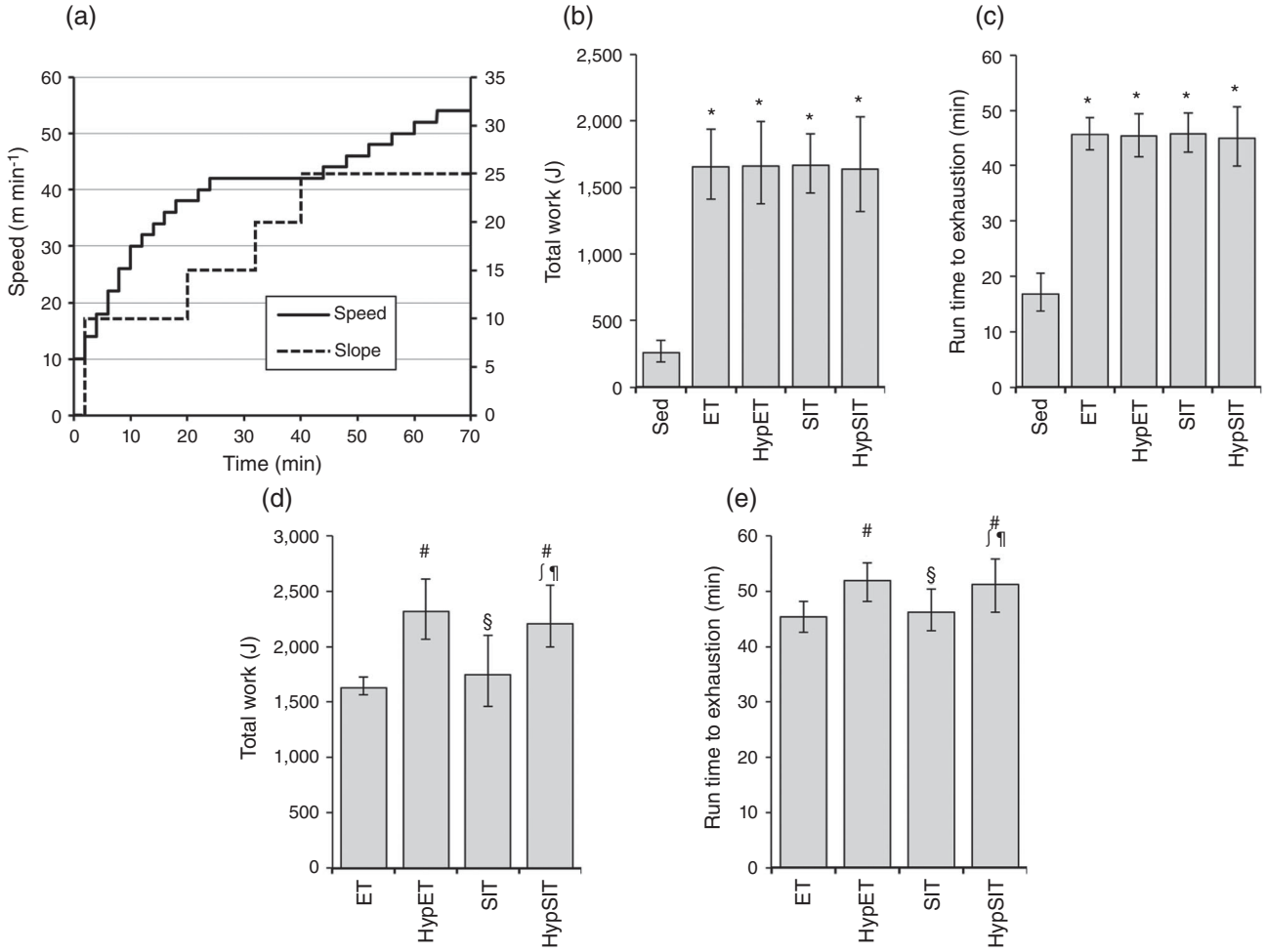

FIGURE 2 Endurance exercise performance test. (a) Graded ramp treadmill running protocol for the endurance capacity test. (b, d) Total work capacity of the endurance capacity test after 7 weeks of voluntary wheel running and after 4 weeks of treadmill exercise training, respectively. (c, e) Run time to exhaustion values after 7 weeks of voluntary wheel running and after 4 weeks of treadmill exercise training, respectively. Sed, sedentary control group; ET, endurance-trained group; HypET, hyperbaric exposure with endurance-trained group; SIT, sprint interval-trained group; HypSIT, hyperbaric exposure with sprint interval-trained group. #Significantly different from pre-treadmill training values of each group shown in panel b or c at $P < 0.05$ using Student's paired *t* test. Significant differences from the *Sed, ⌠ET, §HypET and ¶SIT groups at $P < 0.05$ using the Tukey–Kramer multiple comparisons test. Values are represented as means ± SD

metres per second), time (in seconds), slope (as a percentage) and 9.8 m s^{-2}.

For the endurance test, exhaustion was defined when the mouse stayed for >5 s on the metal grid (no electrical shock) at the rear of the treadmill, despite external gentle touching being applied to the tail with a conventional elastic bamboo stick (0.8 mm in diameter). For the interval test, exhaustion was defined when the mouse could not run three consecutive sprint trials.

After the performance test, mice were given a 48 h non-exercise period before treadmill training. Mice in the training group were divided into an endurance-trained group (ET, $n = 8$), hyperbaric exposure with endurance-trained group (HypET, $n = 8$), sprint interval-trained group (SIT, $n = 9$) or hyperbaric exposure with sprint interval-trained group (HypSIT, $n = 9$), in order to match the mean and SD values of total work (Figures 2b and 3c). Mice in the training groups were subjected to exercise training 6 days per week for 4 weeks using a rodent treadmill (KN-73; Natsume Co., Tokyo, Japan). Instead of using an electrical shock, the tali or planta pedis of mice were touched with a conventional test tube brush made with soft porcine bristles in order to motivate them to run when they stayed on a metal grid for >2 s. In the ET and HypET groups, mice ran for 60 min at 20 m min^{-1} with a 15 deg incline on the first and second days of training. Running speed was increased to 25, 30 and 32.5 m min^{-1} on the first day of the second, third and fourth week, respectively. In the SIT and HypSIT groups, mice were subjected to two sets of the interval exercise regimen (5 sec run–10 s rest) for 25 min interposed by a 10 min rest. Mice ran at 30 m min^{-1} with a 15 deg incline on the first day of training. The running speed was increased to 35, 37.5, 40 and 42.5 m min^{-1} on the fourth, eighth, 11th and 15th day of training, respectively. The maximal endurance capacity test and sprint interval capacity test were performed 48 h after the last run, as described above.

The hyperbaric exposure group was subjected to 1.3 ATA with room air (20.9% O_2) for 1 h, once per day (starting ~30 min after daily exercise), 6 days per week, for 4 weeks. Compressed air (0.2 MPa) was cleaned with an activated charcoal and micromist filter (pore size 0.01 μm; AMF500B-10; Anest Iwata, Co., Yokohama, Japan) and was

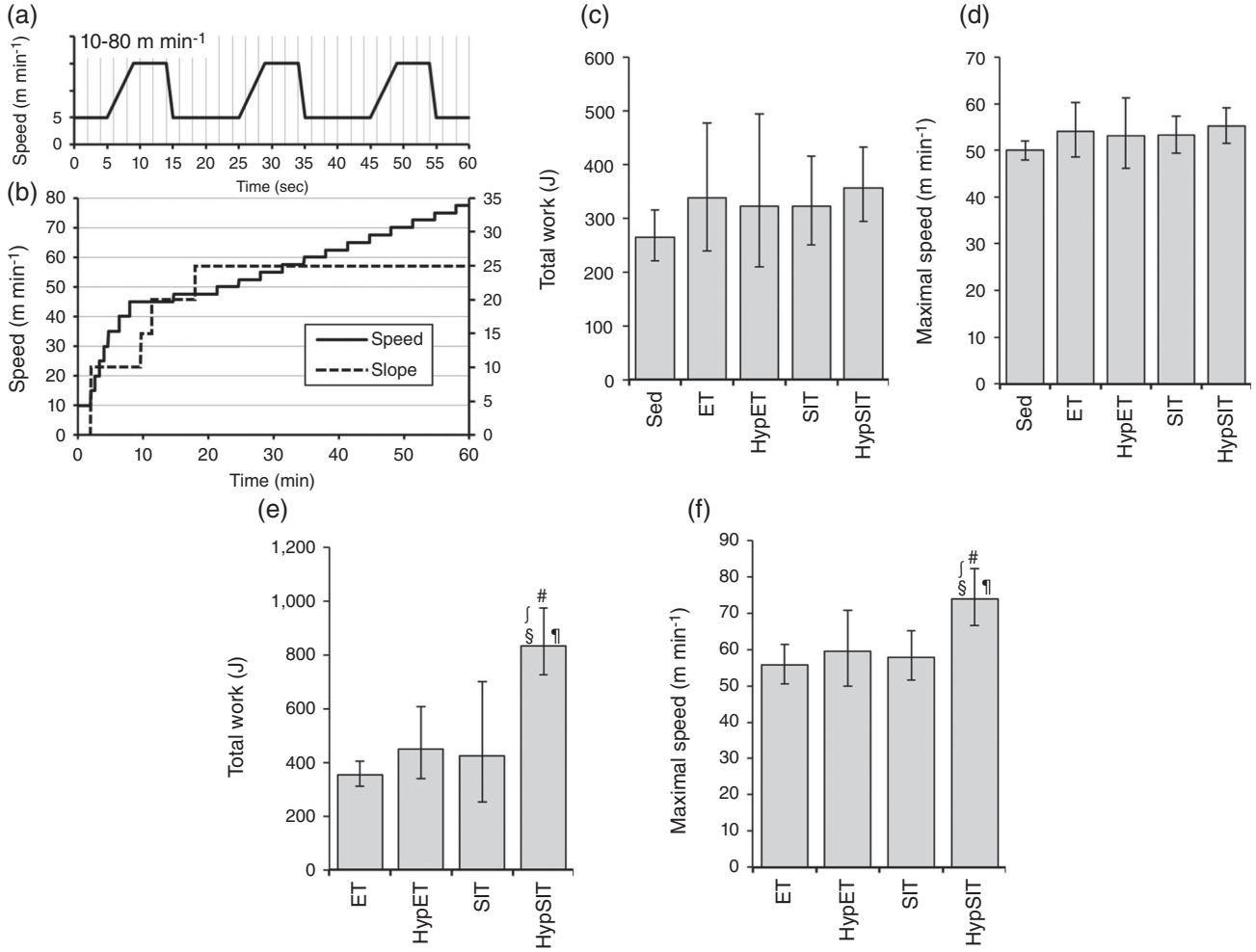

FIGURE 3 Interval exercise performance test. (a) Treadmill running protocol for the interval exercise capacity test. (b) Graded ramp treadmill running protocol for the interval capacity test. (c, e) Total work capacity of the endurance capacity test after 7 weeks of voluntary wheel running and after 4 weeks of treadmill exercise training, respectively. (d, f) Maximal speed values of the interval capacity test after 7 weeks of voluntary wheel running and after 4 weeks of treadmill exercise training, respectively. #Significantly different from pre-treadmill training values of each group shown in panel c or d at $P < 0.05$ using Student's paired *t* test. Significant difference from the ∫ ET, §HypET and ¶SIT groups at $P < 0.05$ using the Tukey–Kramer multiple comparisons test. Values are represented as means ± SD

inflated into the chamber (length 0.65 m, width 0.42 m and height 0.35 m). The pressure in the chamber was monitored using a digital manometer (KDM30; Krone Co., Tokyo, Japan) and was gradually increased or decreased at 0.136 ATA min^{-1} within 2.2 min. During 1 h hyperbaric exposure, compressed air was continuously inflated into the chamber at a rate of 2 l min^{-1} to prevent CO_2 accumulation. The pressure of 1.3 ATA was maintained using a pressure valve (FCAM-B2-A; IBS Co., Osaka, Japan).

Forty-eight hours after the last run, mice were anaesthetized with α-chloralose (0.06 g kg^{-1} I.P., Wako Pure Chemical Industries Ltd., Osaka, Japan) and urethane (0.7 g kg^{-1} I.P., Wako). A toe pinch response was used to validate adequate anaesthesia. The left soleus (SOL), plantaris (PL) and gastrocnemius muscles were excised, and the deep red region (Gr) of the gastrocnemius was isolated from the superficial white region (Gw). The diaphragm (DIA) was excised. All samples were frozen in liquid nitrogen for biochemical analyses. The corresponding muscles on the right side were excised and placed in embedding medium, OCT compound (Miles Inc., Elkhart, IN, USA), and then rapidly frozen in isopentane cooled to its melting point (−160°C) with liquid nitrogen. Mice were killed by excision of the heart. The heart was weighed, and frozen in liquid nitrogen. All tissue samples were stored at −80°C until for analyses.

2.3 | Histological analyses

Histochemical examinations of capillary profiles and muscle fibre phenotypes were done as previously reported, with slight modifications (Suzuki, 2017). Briefly, 10-μm-thick serial cross-sections were obtained using a cryotome (CM-1500; Leica Japan Inc., Tokyo, Japan) at −20°C from the mid-belly portion of the calf muscles. These sections were air dried, fixed with 100% ethanol at 4°C for 15 min, and then washed in 0.1 M phosphate-buffered saline with 0.05% Triton X-100 (PBS-T). Sections were then blocked with 10% normal goat serum at room temperature for 1 h and incubated at 4°C overnight with a mixture of fluorescein-labelled Griffonia simplicifolia lectin [GSL I; FL 1101 (1:300); Vector Laboratories Inc., Burlingame, CA, USA], an

anti-type I myosin heavy chain (MHC) antibody (BA-F8; mouse IgG2b; 1:100) and anti-type IIA MHC antibody (SC-71; mouse IgG1; 1:100) diluted with PBS-T containing 5% goat normal serum. Sections were then reacted with a secondary antibody mixture containing Alexa Fluor 350-labelled anti-mouse IgG2b and Alexa Fluor 647-labelled anti-mouse IgG1 diluted with PBS-T at room temperature for 1 h. Sections were coverslipped with Fluoromount/Plus (K048; Diagnostic BioSystems Co., Pleasanton, CA, USA). Primary and secondary antibodies were purchased from the Developmental Studies Hybridoma Bank (University of Iowa) and Thermo Fisher Scientific Inc. (Tokyo, Japan), respectively. Fluorescent images of the incubated sections were observed using a microscope (Axio Observer; Carl Zeiss Japan, Tokyo, Japan). Muscle fibre phenotypes were classified as type I (blue), type I+IIA (faint blue and faint red), type IIA (red), type IIAX (faint red) and type IIB+IIX (unstained). Non-overlapping microscopic fields were selected at random from each muscle sample. The observer was blinded to the source (groups) of each slide during the measurements. Fluorescent images were obtained from SOL, PL, the lateral (GrL) and medial (GrM) portions of Gr, and Gw. The population of muscle fibres sampled from each muscle or muscle portion ranged from 250 to 350. The negative control without primary antibodies was confirmed to show no fluorescent signal.

A histochemical assessment for PGC-1α expression in the nucleus was performed as reported previously (Suzuki, 2017). Briefly, 10-μm-thick cross-sections were air dried, fixed with 3.8% formaldehyde in PBS for 15 min, and incubated at 4°C overnight with an Alexa Fluor 488-labelled anti-PGC-1α antibody (1:500; Novus Biologicals, Centennial, CO, USA) diluted with PBS-T. After washing with PBS-T, sections were reacted with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; D9542; Sigma-Aldrich, St Louis, MO, USA) to stain the nucleus blue in fluorescent colour. Slides were then rinsed with distilled water and mounted as described above. Two individual images were obtained using the respective filter sets (filter set 49 or filter set 38) at one microscopic field with a constant exposure time for each image and stored on a computer disk. In order to assess the expression of PGC-1α in the nucleus, each RGB image was split into a green (PGC-1α) or blue (nucleus) channel and converted to an eight-bit greyscale image using ImageJ software (NIH, Bethesda, MD, USA). The co-localized area of two images was obtained with a constant threshold value for each image. All fluorescent images were obtained within 24 h of staining.

2.4 | Biochemical analyses of enzyme activities

Frozen tissue powder was obtained using a frozen sample crusher (SK mill; Tokken Inc., Chiba, Japan) and homogenized with ice-cold medium [10 mM Hepes buffer, pH 7.4; 0.1% Triton X-100; 11.5% (w/v) sucrose; and 5% (v/v) protease inhibitor cocktail (P2714; Sigma-Aldrich)]. After centrifugation at 1500*g* at 4°C for 10 min, the supernatant was used in enzyme activity analyses. The activities of 3-hydroxyacyl-CoA-dehydrogenase (HAD) and lactate dehydrogenase (LDH) were assayed according to the method of Bass, Brdiczka, Eyer, Hofer, and Pette (1969). The activities of citrate synthase (CS) and phosphofructokinase (PFK) were assayed according to the method of Srere (1969) and Passonneau and Lowry (1993), respectively. All measurements were conducted at 25°C with a spectrophotometer (U-2001; Hitachi Co., Tokyo, Japan), and enzyme activities were obtained as micromoles per hour per milligram of protein. Total protein concentrations were measured using PRO-MEASURE protein measurement solution (iNtRON Biotechnology Inc., Gyeonggi-do, Korea).

2.5 | Western blot analyses

The tissue homogenates described in section 2.4 were used for Western blot analyses. A sample (50 μg) was fractionated by SDS-PAGE on 7.5 or 12% (w/v) polyacrylamide gels (TGX StainFree FastCast gel; Bio-Rad Inc., Hercules, CA, USA) and electrophoretically transferred to a polyvinylidene fluoride membrane. The bands in each lane on the membrane were detected with an ultraviolet imager (ChemiDoc; Bio-Rad). The blots were blocked with 3% (w/v) bovine serum albumin, 1% (w/v) polyvinylpyrrolidone and 0.3% (v/v) Tween-20 in PBS for 1 h, and then exposed to a specific primary antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) against HSP70 (1:500; sc-66048), Tfam (1:500; sc-166965), Mfn2 (1:500; sc-100560) or Drp1 (1:500; sc-271583) diluted in PBS with 0.05% Tween-20 for 1 h. After the blots had been incubated with an HRP-labelled mouse IgGκ light chain binding protein (1:5000; sc-516102; Santa Cruz), they were reacted with Clarity Western ECL substrate (Bio-Rad), and the required proteins were detected with an imager (ChemiDoc). The densities of the bands were normalized to the densities of all protein bands in each lane on the membrane (Glida & Gomes, 2013; Vigelsø et al., 2014). The densities of the bands were quantified using Image Lab software (Bio-Rad) and normalized to the same sample that was run on every gel and transferred to every membrane.

2.6 | Statistical analyses

All values were $\log_{10}$-transformed. Differences among the four or five groups were examined using the Tukey–Kramer multiple comparisons test. Differences in exercise performance before and after treadmill training with or without hyperbaric exposure were analysed using Student's paired *t* test. Mean, SD and 95% confidence interval (CI) values were expressed after back-transformation. Differences were considered to be significant at $P < 0.05$. When the CI values did not contain the zero value specified in the null hypothesis, differences were considered to be biologically important (du Prel, Hommel, Röhrig, & Blettner, 2009). All statistical analyses were performed using EZR public domain software (Kanda, 2013).

3 | RESULTS

3.1 | Body and organ masses

Body weight was significantly lower in the four exercise-trained groups than in the Sed group (Table 1; $P < 0.05$). The absolute and relative weight values of the whole heart and left ventricle were significantly higher in the four exercise-trained groups than in the Sed group ($P < 0.05$). The relative weight values of gastrocnemius and SOL were

TABLE 1 Values of body and organ masses

	Sed ($n = 10$)	ET ($n = 8$)	HypET ($n = 8$)	SIT ($n = 9$)	HypSIT ($n = 9$)
Body mass (g)	41.4 ± 1.6/1.5	37.4 ± 1.9/1.8*	38.6 ± 2.2/2.1*	38.3 ± 2.5/2.3*	38.8 ± 1.8/1.7*
Organ mass (mg)					
Gastrocnemius	177.6 ± 17.9/16.3	168.6 ± 11.4/10.7	179.4 ± 15.2/14.0	174.9 ± 13.6/12.6	182.7 ± 14.4/13.4
Soleus	10.0 ± 0.82/0.76	9.7 ± 1.3/1.1	10.1 ± 1.2/1.1	10.4 ± 0.62/0.58	10.7 ± 1.4/1.3
Plantaris	23.8 ± 2.4/2.1	23.1 ± 1.6/1.5	24.3 ± 3.2/2.8	24.2 ± 2.4/2.2	24.3 ± 2.7/2.4
Whole heart	157.0 ± 8.0/7.5	173.6 ± 13.0/12.1*	184.7 ± 12.0/11.3*	180.6 ± 13.2/12.3*	185.8 ± 10.9/10.3*
Left ventricle	111.8 ± 7.1/6.6	126.6 ± 8.6/8.1*	134.5 ± 9.3/8.7*	131.5 ± 8.4/7.9*	134.9 ± 8.9/8.4*
Organ mass-to-body mass ratio (mg g^{-1})					
Gastrocnemius	4.28 ± 0.43/0.39	4.51 ± 0.09/0.09	4.65 ± 0.32/0.30	4.57 ± 0.27/0.25	4.74 ± 0.24/0.23*
Soleus	0.24 ± 0.02/0.02	0.26 ± 0.02/0.02	0.26 ± 0.04/0.03	0.27 ± 0.02/0.02	0.28 ± 0.04/0.03
Plantaris	0.57 ± 0.06/0.06	0.62 ± 0.04/0.03	0.64 ± 0.07/0.06	0.63 ± 0.06/0.06	0.63 ± 0.08/0.07
Whole heart	3.79 ± 0.20/0.19	4.65 ± 0.17/0.17*	4.78 ± 0.14/0.14*	4.72 ± 0.34/0.32*	4.79 ± 0.30/0.29*
Left ventricle	2.70 ± 0.13/0.12	3.39 ± 0.13/0.13*	3.48 ± 0.11/0.10*	3.44 ± 0.19/0.18*	3.48 ± 0.25/0.24*

Values are represented as means ± SD (upper/lower). Sed, sedentary control group; ET, endurance-trained group; HypET, hyperbaric exposure with endurance-trained group; SIT, sprint interval-trained group; HypSIT, hyperbaric exposure with sprint interval-trained group. *Significantly different from the Sed group at $P < 0.05$ using Tukey–Kramer multiple comparison.

markedly higher in the HypSIT group (CI: 1.09–1.12, $P = 0.017$ and CI: 1.11–1.18, $P = 0.052$, respectively) than in the Sed group. The relative weight values of gastrocnemius were markedly higher in the HypET group than in the Sed group (CI: 1.07–1.10, $P = 0.089$). Thus, hyperbaric exposure facilitated muscle hypertrophy induced by endurance and interval exercise training.

3.2 | Maximal exercise capacity

After voluntary wheel running for 7 weeks, total work and run time to exhaustion values were significantly greater in the training group by 6.4-fold (Figure 2b; CI: 6.05–6.80, $P < 0.001$) and 2.7-fold (Figure 2c; CI: 2.68–2.78, $P < 0.01$), respectively, than in the Sed group. In contrast, total work and maximal speed values of the interval exercise test were slightly greater in the training group by 1.3-fold (Figure 3c; CI: 1.14–1.41, $P = 0.13$) and by 1.3-fold (Figure 3d; CI: 1.14–1.41, $P = 0.13$), respectively, than in the Sed group. ET and SIT for 4 weeks increased neither endurance (total work and run time to exhaustion; Figure 2d and e, respectively) nor interval exercise capacity (total work and maximal speed; Figure 3e and f, respectively). HypET significantly increased total work (1.4-fold, CI: 1.24–1.56, $P < 0.001$) and run time (1.1-fold, CI: 1.09–1.20, $P < 0.01$) values for the endurance capacity test. HypSIT markedly enhanced total work (1.3-fold, CI: 1.20–1.51, $P < 0.001$) and run time (1.1-fold, CI: 1.07–1.21, $P < 0.001$) values for the endurance test and total work (2.3-fold, CI: 1.84–2.97, $P < 0.001$) and maximal speed (1.3-fold, CI: 1.24–1.45, $P < 0.001$) for the interval test. Moreover, total work and run time values for the endurance test were significantly greater in the HypET group than in the ET group (CI: 1.26–1.59, $P = 0.0015$ and CI: 1.09–1.20, $P = 0.009$, respectively) and were greater in the HypSIT group than in the SIT group (CI: 1.13–1.41, $P = 0.026$ and CI: 1.18–1.38, $P = 0.040$, respectively). Total work and maximal speed values for the interval test were markedly greater in the HypSIT group than in other training groups ($P < 0.05$). Thus, hyperbaric exposure had additive effects on exercise-induced improvements in both endurance and interval exercise capacities.

3.3 | Fibre type composition

Representative immunofluorescent images of SOL are shown in Figure 4. The proportion of type I fibres in SOL was markedly greater in the HypSIT group than in the SIT (CI: 1.12–1.18, $P = 0.069$) and HypET (CI: 1.12–1.19, $P = 0.069$) groups (Table 2). The proportion of type IIA fibres in PL was significantly greater in the HypET group (CI: 1.22–1.31, $P = 0.027$), but not in the ET group (CI: 1.13–1.22, $P = 0.23$), compared with the Sed group. The proportion of type IIAX fibres in PL and GrL was significantly greater in the four trained groups than in the Sed group ($P < 0.05$). The proportion of type IIAX fibres in GrL was significantly greater in the ET (CI: 3.47–4.47, $P = 0.025$) and HypET (CI: 2.14–3.65, $P = 0.024$) groups than in the Sed group. The proportion of type IIA fibres in GrM was markedly greater in the ET (CI: 1.27–1.34, $P = 0.025$), SIT (CI: 1.26–1.36, $P = 0.018$) and HypSIT (CI: 1.19–1.34, $P = 0.048$) groups than in the Sed group. Thus, hyperbaric exposure increased the proportion of highly oxidative muscle fibres in interval-trained hindleg muscles.

3.4 | Fibre cross-sectional area

The fibre cross-sectional area (FCSA) values of type I fibres in SOL were markedly higher in the HypSIT group than in the SIT group (CI: 1.16–1.25, $P = 0.071$; Table 3). The FCSA values in PL were markedly higher in the HypET group than in the Sed and ET groups for type IIA (CI: 1.25–1.44, $P = 0.038$ and CI: 1.16–1.34, $P = 0.23$, respectively), IIAX (CI: 1.30–1.52, $P = 0.026$ and CI: 1.17–1.37, $P = 0.25$, respectively) and IIB+IIX fibres (CI: 1.16–1.34, $P = 0.14$ and CI: 1.16–1.34, $P = 0.17$, respectively). Moreover, FCSA values in PL were markedly higher in the HypSIT group than in the Sed group for type I (CI: 1.24–1.49, $P = 0.031$), IIAX (CI: 1.29–1.46, $P = 0.037$) and IIB+IIX (CI: 1.21–1.34, $P = 0.066$) fibres, whereas these values were markedly higher in the

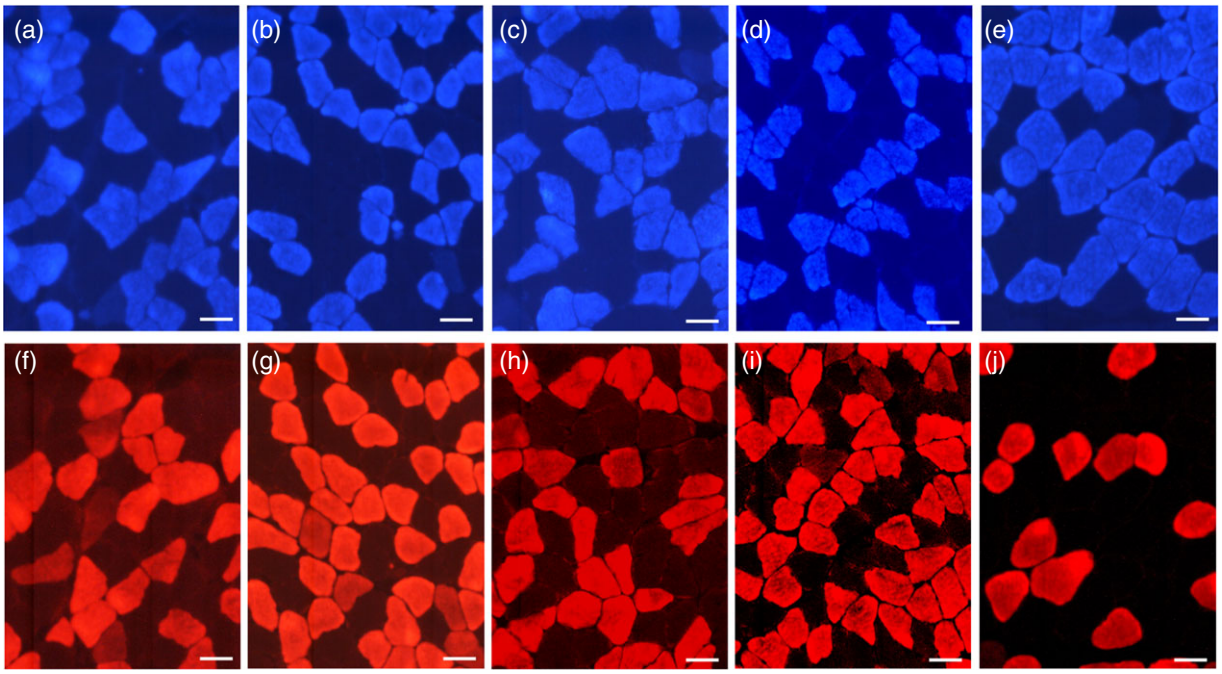

FIGURE 4 Representative immunofluorescent images of the soleus muscle: (a–e) type I fibre (blue); (f–j) type IIA fibre (red); (a, f) Sed group; (b, g) ET group; (c, h) HypET group; (d, i) SIT group; and (e, j) HypSIT group. Scale bars represent 30 μm

TABLE 2 Fibre type composition values (percentages)

	Type	Sed	ET	HypET	SIT	HypSIT
SOL	I	54.2 ± 3.9/3.6	54.0 ± 5.4/4.9	52.1 ± 3.4/3.2	52.4 ± 9.5/8.1	60.3 ± 6.6/5.9
	IIA	43.0 ± 4.2/3.9	43.8 ± 5.4/4.8	46.0 ± 3.0/2.8	42.0 ± 11.1/8.8	36.9 ± 6.7/5.7§
	IIAX	1.4 ± 2.7/1.1	1.1 ± 1.4/0.74	0.92 ± 1.5/0.72	1.6 ± 3.1/1.3	0.62 ± 1.4/0.61
PL	I	5.8 ± 4.3/2.6	3.4 ± 7.2/2.5	3.5 ± 6.1/2.4	5.2 ± 6.1/2.6	2.2 ± 5.7/1.8
	IIA	43.2 ± 6.0/5.3	50.7 ± 8.7/7.4	54.7 ± 8.4/7.3*	48.6 ± 9.1/7.7	51.0 ± 10.6/8.8
	IIAX	3.1 ± 2.9/1.6	15.7 ± 5.0/3.8*	13.6 ± 9.5/5.7*	10.2 ± 9.5/5.7*	11.9 ± 5.9/4.0*
	IIB+IIX	45.4 ± 7.7/6.6	25.5 ± 9.2/6.8*	23.1 ± 9.6/6.8*	31.0 ± 5.9/5.0*	30.0 ± 11.4/8.3*
GrL	I	18.0 ± 3.4/2.8	17.5 ± 2.2/1.9	17.9 ± 3.0/2.6	18.4 ± 1.1/1.0	17.6 ± 3.7/3.0
	IIA	44.4 ± 4.2/3.8	51.2 ± 8.6/7.4*	50.4 ± 7.4/6.5	54.0 ± 5.4/5.0*	49.3 ± 7.0/6.1
	IIAX	2.5 ± 3.2/1.5	9.7 ± 5.9/3.7*	6.9 ± 11.5/4.5*	7.8 ± 3.3/2.4*	10.3 ± 5.4/3.6*
	IIB+IIX	33.6 ± 5.4/4.7	17.7 ± 12.6/7.4*	20.3 ± 10.5/7.0	17.6 ± 11.0/6.8	18.5 ± 14.2/8.1
GrM	I	44.4 ± 8.3/7.0	44.0 ± 6.2/5.4	47.7 ± 7.4/6.4	44.0 ± 7.1/6.1	41.1 ± 5.9/5.2
	IIA	35.2 ± 7.9/6.4	45.8 ± 5.2/4.7*	41.6 ± 4.7/4.2	45.9 ± 8.4/7.1*	44.5 ± 12.3/9.6*
	IIAX	2.8 ± 3.8/1.8	3.3 ± 7.1/2.5	2.9 ± 6.9/2.3	4.4 ± 6.5/2.8	6.0 ± 9.2/3.8
	IIB+IIX	14.1 ± 7.9/5.1	3.1 ± 6.0/2.2*	3.3 ± 5.8/2.3*	1.5 ± 4.4/1.4*	2.2 ± 6.7/1.9*
Gw	IIB+IIX	100	100	100	100	100

Values are represented as means ± SD (upper/lower). SOL, soleus muscle; PL, plantaris muscle; GrL, lateral postion of red gastrocnemius muscle; GrM, medial portion of red gastrocnemius muscle; Gw, white portion of gastrocnemius muscle. Significant differences from the *Sed and §HypET groups, at $P < 0.05$ using the Tukey–Kramer multiple comparisons test.

SIT group than in the Sed group for type I fibres only (CI: 1.22–1.35, $P = 0.054$). Thus, hyperbaric exposure promoted muscle fibre hypertrophy in intermediate and glycolytic fibres after endurance training and in oxidative and glycolytic fibres after interval training.

3.5 | Capillarization

Capillary-to-fibre (C:F) ratio values in GrL were significantly higher in the HypET group, but not in the ET group, compared with the Sed group (CI: 1.30–1.37, $P = 0.006$; Table 4). In PL and GrM, C:F values were significantly higher in the four trained groups than in the Sed group ($P < 0.05$). The C:F and capillary density values in Gw were markedly higher in the HypSIT group than in the SIT group (CI: 1.21–1.36, $P = 0.049$ and CI: 1.17–1.40, $P = 0.104$, respectively). Thus, in interval-trained mice, daily hyperbaric exposure facilitated exercise-induced capillary growth in the muscle regions mainly composed of glycolytic fibres.

TABLE 3 Fibre cross-sectional area values (in square micrometres)

	Type	Sed	ET	HypET	SIT	HypSIT
SOL	I	1798.6 ± 232.9/206.2	1607.0 ± 317.9/265.4	1525.9 ± 204.7/180.5	1547.9 ± 229.6/199.9	1866.1 ± 284.6/246.9
	IIA	1806.7 ± 219.7/195.8	1688.6 ± 334.7/279.3	1727.9 ± 338.1/282.8	1713.0 ± 365.7/307.4	1893.1 ± 213.1/191.6
	IIAX	1925.1 ± 429.6/351.2	1811.6 ± 611.2/457.0	1748.1 ± 515.9/398.4	1928.6 ± 365.7/307.4	2057.2 ± 880.7/616.7
PL	I	1024.9 ± 227.5/186.2	1152.3 ± 173.5/150.8	1164.2 ± 184.8/159.1	1319.2 ± 314.0/253.7	1393.8 ± 389.8/304.6*
	IIA	1277.4 ± 175.8/154.5	1374.0 ± 235.3/200.9	1711.3 ± 534.9/407.5*	1573.3 ± 447.4/348.3	1653.7 ± 372.2/303.8
	IIAX	1907.6 ± 447.6/362.5	2113.2 ± 319.6/277.6	2683.2 ± 927.6/689.3*	2485.5 ± 613.9/492.3	2615.2 ± 763.8/591.1*
	IIB+IIX	2374.1 ± 447.6/362.5	2368.1 ± 392.1/336.4	2955.7 ± 915.2/698.8	2836.0 ± 661.2/536.2	3026.5 ± 706.1/572.5
GrL	I	1452.9 ± 466.6/353.2	1345.8 ± 599.0/414.5	1434.8 ± 387.7/305.2	1428.3 ± 322.7/263.3	1393.0 ± 481.2/357.7
	IIA	1586.5 ± 534.5/399.8	1518.6 ± 605.9/433.1	1719.1 ± 516.3/397.1	1439.1 ± 352.8/283.3	1555.6 ± 521.4/390.5
	IIAX	2229.1 ± 547.8/439.7	2267.7 ± 805.4/594.3	2546.6 ± 839.3/631.2	2211.1 ± 372.3/318.6	2294.0 ± 796.9/591.4
	IIB+IIX	3167.4 ± 547.8/439.7	2796.1 ± 1187.1/833.3	2969.8 ± 1213.7/861.6	2720.9 ± 584.0/480.8	2811.3 ± 841.1/647.4
GrM	I	1772.0 ± 389.0/319.0	1654.5 ± 182.9/164.7	1792.1 ± 389.6/320.0	1883.8 ± 247.2/218.5	1996.2 ± 513.7/408.6
	IIA	1549.8 ± 303.0/253.4	1547.6 ± 237.2/205.7	1754.6 ± 412.0/333.6	1573.5 ± 189.0/168.7	1819.4 ± 551.8/423.4
	IIAX	1976.8 ± 253.8/224.9	1925.1 ± 124.7/117.2	2008.0 ± 250.2/222.4	2131.7 ± 318.0/276.7	2204.8 ± 309.4/271.3
	IIB+IIX	2246.0 ± 348.4/301.6	2102.6 ± 305.0/266.4	2192.0 ± 427.6/357.8	2455.1 ± 360.0/313.9	2136.4 ± 292.6/257.4
Gw	IIB+IIX	2895.8 ± 587.9/488.7	2623.7 ± 318.6/284.1	2771.0 ± 413.0/359.5	2751.8 ± 310.5/279.0	2949.3 ± 422.4/384.7

Values are represented as means ± SD (upper/lower). *Significantly different from the Sed group at $P < 0.05$ using the Tukey–Kramer multiple comparisons test.

TABLE 4 Capillary-to-fibre ratio and capillary density values

	Sed	ET	HypET	SIT	HypSIT
Capillary-to-fibre ratio					
SOL	1.85 ± 0.19/0.18	1.89 ± 0.12/0.11	1.85 ± 0.14/0.13	1.87 ± 0.15/0.14	1.90 ± 0.07/0.07
PL	1.74 ± 0.12/0.11	2.06 ± 0.15/0.14*	2.05 ± 0.32/0.28*	2.15 ± 0.21/0.19*	2.16 ± 0.24/0.22*
GrL	1.83 ± 0.27/0.24	2.17 ± 0.41/0.35	2.29 ± 0.24/0.22*	2.24 ± 0.16/0.15*	2.31 ± 0.38/0.33*
GrM	1.72 ± 0.28/0.25	2.11 ± 0.25/0.23*	2.20 ± 0.49/0.42*	2.12 ± 0.24/0.22*	2.14 ± 0.21/0.20*
Gw	1.00 ± 0.17/0.15	1.02 ± 0.20/0.18	1.10 ± 0.14/0.13	0.91 ± 0.17/0.15	1.17 ± 0.31/0.26¶
Capillary density (number mm^{-2})					
SOL	1097.6 ± 214.6/179.5	1152.5 ± 179.5/155.3	1204.9 ± 137.0/1236.0	1205.8 ± 147.8/131.7	1124.6 ± 142.8/126.8
PL	1062.3 ± 115.5/104.2	1283.9 ± 152.5/136.3*	1046.7 ± 132.5/136.3/	1184.6 ± 229.5/192.3	1127.9 ± 141.6/125.8
GrL	965.2 ± 261.6/205.9	1302.8 ± 322.4/258.5*	1161.7 ± 312.8/246.5*	1379.5 ± 166.3/148.4	1391.1 ± 212.8/184.6*
GrM	1010.5 ± 247.3/198.7	1271.7 ± 230.7/195.3*	1253.6 ± 177.5/155.5*	1188.3 ± 126.2/114.1	1219.3 ± 241.3/201.4
Gw	434.6 ± 57.2/50.6	471.9 ± 88.9/74.8	486.4 ± 57.1/51.1	398.4 ± 53.7/47.3	505.4 ± 209.0/147.9

Values are represented as means ± SD (upper/lower). Significant differences from the *Sed, /ET and ¶SIT groups, at $P < 0.05$ using the Tukey–Kramer multiple comparisons test.

3.6 | Metabolic enzyme activities

Enzyme activity values in Gr were markedly higher in the HypET group than in the ET group for CS (CI: 1.08–1.29, $P = 0.072$), HAD (CI: 1.10–1.24, $P = 0.036$) and PFK (CI: 2.08–2.68, $P = 0.003$; Table 5). CS activity values in DIA were markedly higher in the HypET (CI: 1.24–1.51, $P < 0.001$) and HypSIT (CI: 1.11–1.42, $P = 0.007$) groups than in the Sed group. PFK activity values in SOL were significantly lower in the HypET group than in the Sed (CI: 0.31–0.94, $P = 0.015$) and ET groups (CI: 0.39–1.18, $P = 0.006$). LDH activity values in PL were markedly higher in the HypSIT group than in the SIT group (CI: 1.09–1.23, $P = 0.008$). CS activity values in SOL were markedly higher in the SIT group than in the HypSIT group (CI: 1.12–1.29, $P = 0.031$). Moreover, PFK activity values in Gw were markedly higher in the SIT group than in the HypSIT group (CI: 1.11–1.25, $P = 0.056$). Thus, HypET enhanced mitochondrial oxidative and glycolytic enzyme activities, whereas HypSIT enhanced the activities of enzymes involved in lactate metabolism in the hindleg muscles.

3.7 | PGC-1α expression

Representative fluorescent images of PL are shown in Figure 5. The PGC-1α-positive nuclear area was significantly ($P < 0.05$) larger in all four training groups in SOL (Figure 6a) and GrL (Figure 6c). PGC-1α expression levels in PL were markedly higher in the HypET group than in the Sed (23.1-fold, CI: 18.7–28.6, $P = 0.002$) and ET groups (2.2-fold, CI: 1.78–2.74, $P = 0.83$; Figure 6b). Moreover, PGC-1α levels were markedly higher in the HypSIT group than in the Sed (14.2-fold,

TABLE 5 Enzyme activity values (in micromoles per hour per milligram of protein)

		Sed	ET	HypET	SIT	HypSIT
CS	SOL	9.9 ± 1.9/1.6	11.0 ± 2.5/2.1	11.0 ± 1.9/1.6	12.7 ± 134/1.2*	10.1 ± 1.6/1.4¶
	PL	7.8 ± 1.5/1.2	9.6 ± 0.73/0.68*	10.4 ± 1.2/1.1*	10.5 ± 1.4/1.3*	9.8 ± 1.4/1.2*
	Gr	13.5 ± 1.4/1.3	17.2 ± 1.5/1.4*	20.3 ± 2.5/2.2*	17.4 ± 3.4/2.9*	17.8 ± 1.8/1.7*
	Gw	5.4 ± 0.6/0.6	6.5 ± 2.3/1.7	6.4 ± 2.0/1.5	6.5 ± 1.1/0.95	6.4 ± 1.0/0.88
	DIA	16.0 ± 2.1/1.9	18.8 ± 3.1/2.6	22.0 ± 3.1/2.7*	18.2 ± 1.5/1.3§	20.1 ± 3.9/3.2*
HAD	SOL	2.6 ± 0.54/0.45	2.4 ± 0.53/0.43	2.2 ± 0.36/0.31	2.8 ± 0.53/0.44§	2.4 ± 0.30/0.27
	PL	1.6 ± 0.31/0.25	1.7 ± 0.30/0.26	1.8 ± 0.22/0.20	1.6 ± 0.33/0.28	1.6 ± 0.09/0.08
	Gr	2.7 ± 0.29/0.26	3.5 ± 0.24/0.22*	4.0 ± 0.34/0.31*ʃ	3.7 ± 0.49/0.43*	3.4 ± 0.43/0.38*§
	Gw	0.92 ± 0.09/0.09	1.1 ± 0.14/0.13*	1.2 ± 0.16/0.14*	1.1 ± 0.12/0.11*	1.1 ± 0.08/0.07*
	DIA	9.9 ± 1.7/1.5	12.1 ± 1.5/1.3*	12.2 ± 1.3/1.2*a	10.8 ± 1.0/0.92	11.2 ± 1.3/1.1
LDH	SOL	67.5 ± 7.5/6.7	42.1 ± 6.8/5.8*	44.5 ± 3.3/3.1*	50.0 ± 8.1/6.9*ʃ	48.6 ± 5.2/4.7*
	PL	139.0 ± 12.6/11.5	112.5 ± 10.7/9.7*	125.1 ± 10.7/9.8	120.9 ± 10.8/9.9*	139.7 ± 12.8/11.7ʃ¶
	Gr	77.1 ± 7.1/6.5	67.3 ± 7.1/6.4	63.0 ± 6.1/5.5*	65.3 ± 9.7/8.4*	71.6 ± 7.0/6.4
	Gw	215.0 ± 27.3/24.2	201.5 ± 14.5/13.6	217.1 ± 9.9/9.5	210.4 ± 18.0/16.6	221.5 ± 15.0/14.0
	DIA	98.0 ± 10.8/9.7	80.6 ± 11.6/10.1*	84.7 ± 6.7/6.2*	82.4 ± 7.6/6.9*	88.4 ± 7.2/6.7
PFK	SOL	3.1 ± 3.6/1.7	2.5 ± 2.4/1.2	1.7 ± 1.8/0.88*ʃ	3.2 ± 3.7/1.8§	3.3 ± 3.4/1.7§
	PL	5.4 ± 4.3/2.4	4.5 ± 2.2/1.5	5.0 ± 1.3/1.1	5.6 ± 1.8/1.4	5.1 ± 0.97/0.82
	Gr	6.8 ± 1.5/1.3	5.7 ± 3.9/2.3	13.6 ± 2.5/2.1*ʃ	6.4 ± 5.4/2.9	5.5 ± 3.6/2.2§
	Gw	11.1 ± 1.9/1.6	9.6 ± 1.9/1.6	11.2 ± 2.2/1.8	14.3 ± 2.0/1.7*ʃ§	11.6 ± 1.5/1.3
	DIA	9.5 ± 1.1/1.0	10.0 ± 1.0/0.9	10.3 ± 1.6/1.4	10.3 ± 1.4/1.3	9.4 ± 1.2/1.1

Values are represented as means ± SD (upper/lower). Abbreviations: CS, citrate synthase; HAD, 3-hydroxyacyl-CoA-dehydrogenase; LDH, lactate dehydrogenase; and PFK, phosphofructokinase. Gr, red portion of gastrocnemius muscle. Significant differences from the *Sed, ʃET, §HypET and ¶SIT groups, at $P < 0.05$ using the Tukey–Kramer multiple comparisons test.

CI: 11.6–17.4, $P = 0.013$) and SIT (2.7-fold, CI: 2.17–3.27, $P = 0.64$) groups. PGC-1α expression levels in GrM were markedly higher in the HypET group than in the Sed (30.2-fold, CI: 26.2–49.3, $P = 0.003$) and ET (6.7-fold, CI: 4.25–10.56, $P = 0.26$) groups (Figure 6d). PGC-1α levels in Gw were significantly higher in the HypSIT group (37.9-fold, CI: 22.9–62.7, $P = 0.035$) than in the Sed group (Figure 6e). Thus, chronic intermittent hyperbaric exposure facilitated PGC-1α protein expression in the nuclei of the hindleg muscles.

3.8 | Protein expression

HSP70 protein expression in all hindleg muscles was significantly stronger in the four training groups than in the Sed group (Figure 7a–e; $P < 0.05$). HSP70 expression in Gr was markedly stronger in the HypET group than in the ET group (2.7-fold, CI: 1.38–5.10, $P = 0.47$). Thus, hyperbaric exposure had an additive effect on HSP70 expression for ET, but not for SIT.

Tfam protein expression in PL, Gr, Gw and DIA was markedly stronger in the four training groups than in the Sed group (Figure 7g–j). Tfam protein expression in Gr was markedly greater in the HypET group than in the ET group (1.3-fold, CI: 1.15–1.56, $P = 0.19$; Figure 7h). Tfam levels in Gw were significantly higher in the HypET group (1.8-fold, CI: 1.25–2.65, $P = 0.035$), but slightly higher in the ET group (1.4-fold, CI: 1.05–1.89, $P = 0.43$) than in the Sed group (Figure 7i).

Drp1 protein expression in Gr and DIA was significantly stronger in the four training groups than in the Sed group ($P < 0.05$; Figure 8c, e). Drp1 protein expression in Gr was markedly stronger, by 1.4-fold, in the HypET group than in the ET group (CI: 1.08–1.70, $P = 0.47$; Figure 8c). Drp1 levels in Gw were significantly higher, by 2.0-fold, in the HypET group (CI: 1.59–2.63, $P = 0.008$), but slightly higher in the ET group (CI: 1.16–2.14, $P = 0.18$), than in the Sed group (Figure 8d). Thus, hyperbaric exposure had an additive effect on Tfam and Drp1 expression for ET, but not for SIT.

Mfn2 protein expression in Gw was markedly stronger in the four training groups than in the Sed group (Figure 8i; $P < 0.05$). Mfn2 levels were significantly higher in the SIT group than in the Sed group in SOL (1.3-fold, CI: 1.25–1.43, $P = 0.009$) and PL (1.6-fold, CI: 1.35–1.89, $P = 0.033$). Thus, hyperbaric exposure did not affect Mfn2 protein expression in hindleg muscles.

4 | DISCUSSION

In the present study, 7 weeks of voluntary wheel running markedly increased total work (6.4-fold; Figure 2b) and run time to exhaustion (2.7-fold; Figure 2c) values in the endurance performance test. However, in the interval performance test, total work (1.3-fold; Figure 3c) and maximal speed (1.1-fold; Figure 3d) values were not improved by the wheel running. Wheel running activity was shown to be an interval exercise (Houle-Leroy, Garland, Swallow, & Guderley, 2000). However, wheel running distance was shown to have a positive

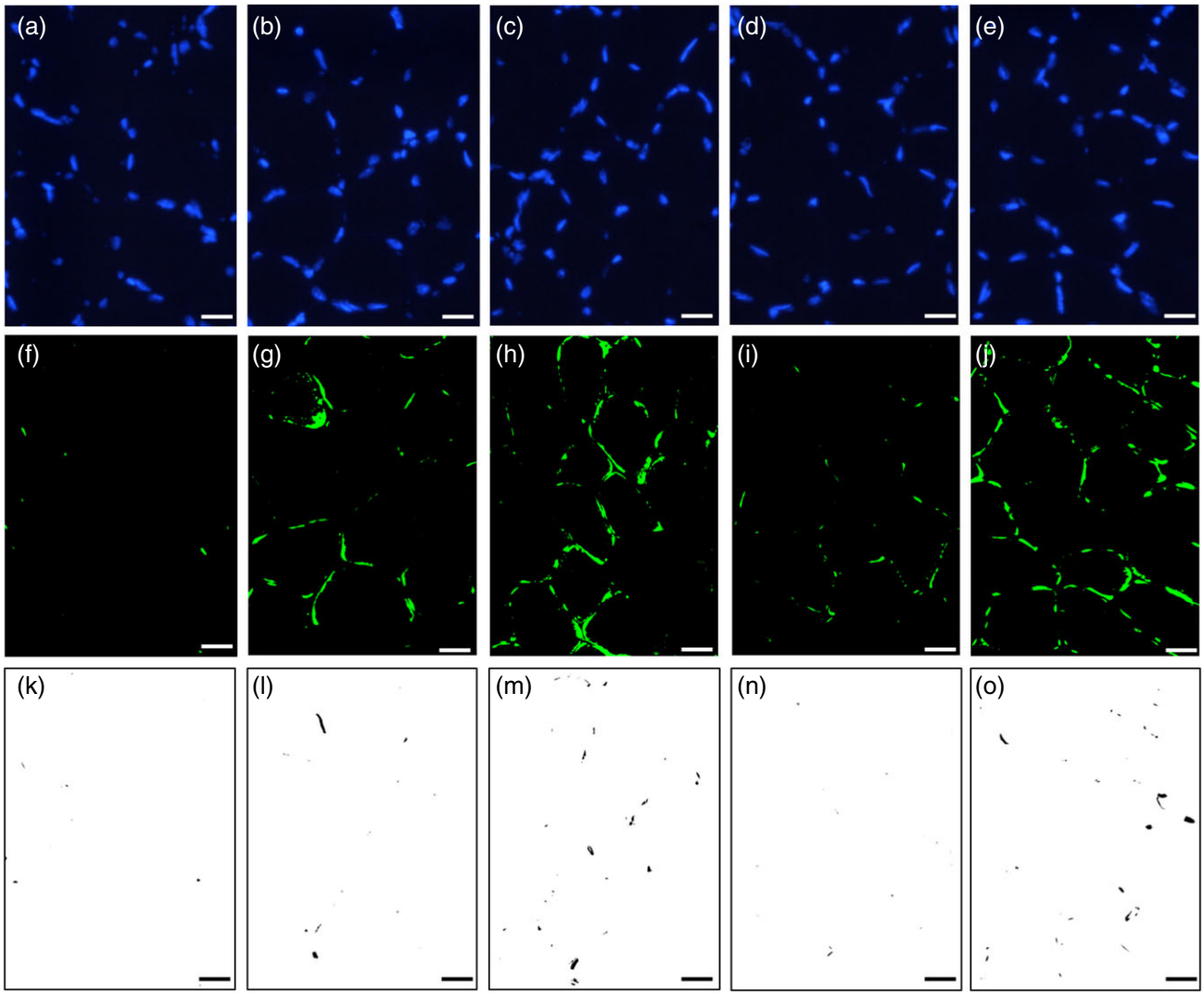

FIGURE 5 Representative immunofluorescent images of the plantaris muscle. (a–e) Immunofluorescent images of the nucleus. (f–j) Immunofluorescent images of PGC-1α. (k–o) Images of co-localized area. (a, f, k) Sed group. (b, g, l) ET group. (c, h, m) HypET group. (d, i, n) SIT group. (e, j, o) HypSIT group. Scale bars represent 20 μm

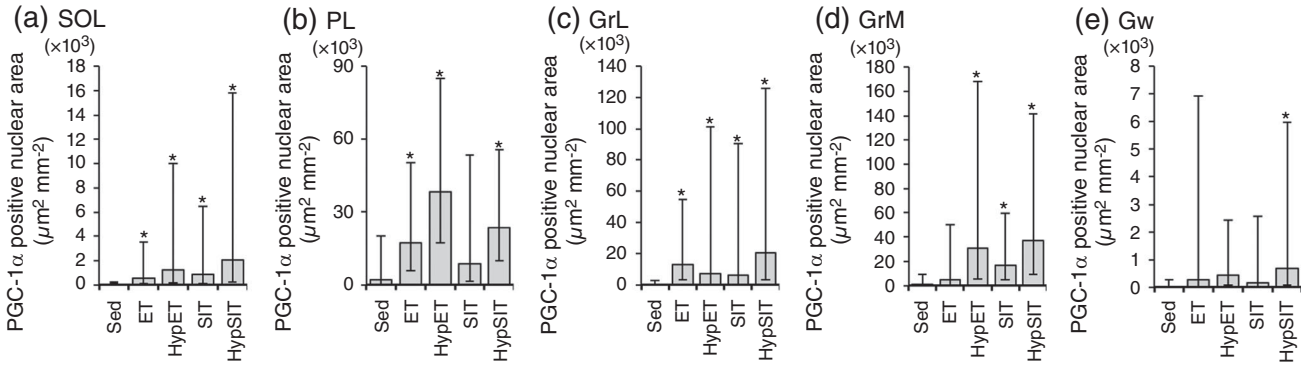

FIGURE 6 Histochemical identification of PGC-1α expression in the nucleus. Co-localized area values for PGC-1α and the nucleus. Values are represented as means ± SD. SOL, soleus muscle; PL, plantaris muscle; GrL, lateral postion of red gastrocnemius muscle; GrM, medial portion of red gastrocnemius muscle; Gw, white portion of gastrocnemius muscle. *Significantly different from the Sed group at $P < 0.05$ using the Tukey–Kramer multiple comparisons test

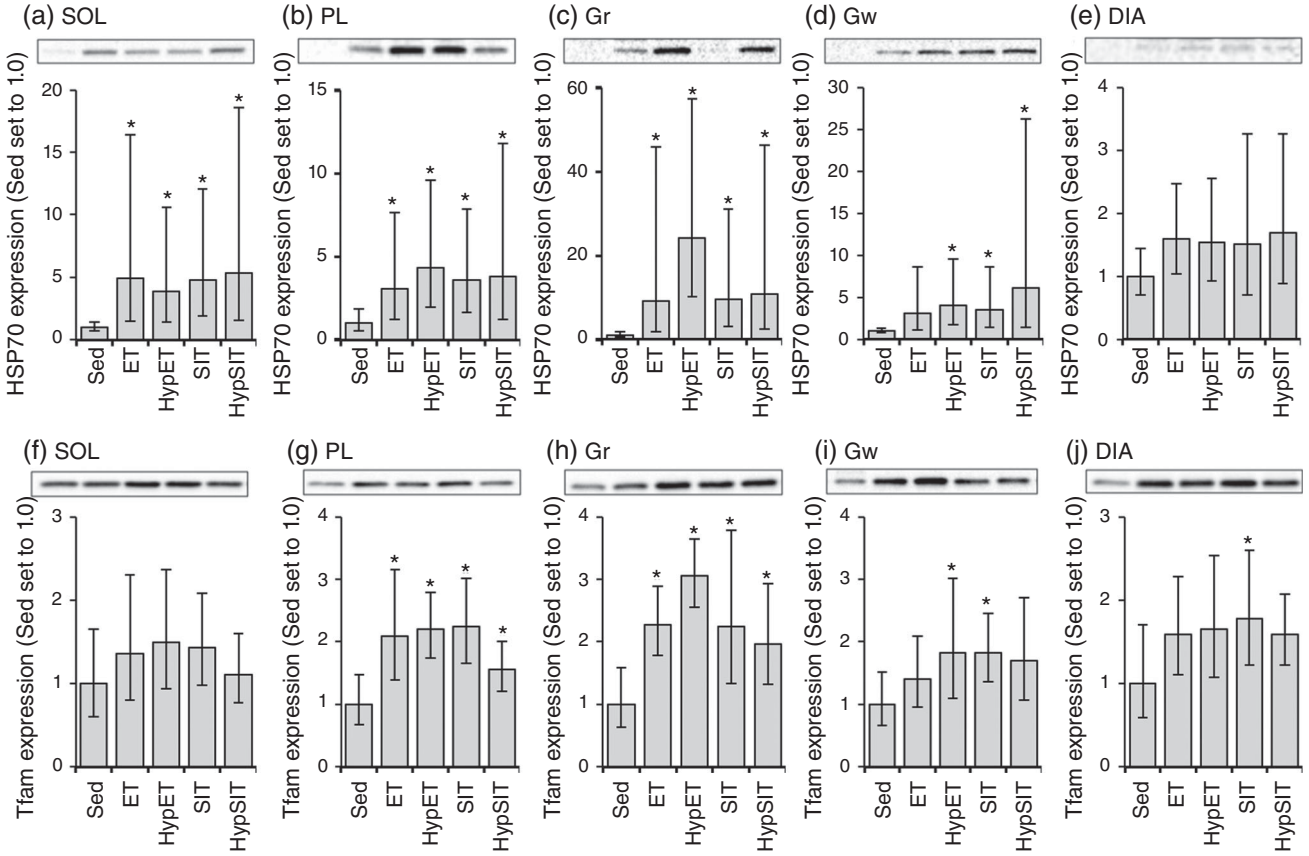

FIGURE 7 Expression of HSP70 (a–e) and Tfam (f–j) proteins. Values are represented as means ± SD. *Significantly different from the Sed group at $P < 0.05$ using the Tukey–Kramer multiple comparisons test

relationship to maximal endurance capacity on the treadmill (Meek, Lonquich, Hannon, & Garland, 2009) and to CS activity values (Ivarsson et al., 2017). Thus, chronic voluntary wheel running predominantly improves endurance capacity.

4.1 | Effects of hyperbaric exposure on endurance training

In highly trained mice, the present results showed that endurance exercise performance was promoted by HypET. The present study demonstrated that expression of the PGC-1α protein expression in the nucleus markedly increased after HypET (Figures 5 and 6). This result is consistent with previous findings on untrained mice; higher PGC-1α and Tfam levels were observed after 4 weeks of endurance training with, but not without, hyperbaric exposure (Suzuki, 2017). In the present study using highly trained mice, Tfam levels in PL, Gr, Gw and DIA markedly increased in the four training groups irrespective of the exercise type and hyperbaric exposure (Figure 7). However, in Gr, the ET-induced upregulation of Tfam was facilitated by hyperbaric exposure (Figure 7h). Tfam expression is mainly regulated by PGC-1α, and a complex of these two proteins promoted mitochondrial biogenesis by the transcription of mitochondrial genome-encoded genes and mitochondrial DNA replication (Wu et al., 1999). After HypET, the activity levels of mitochondrial enzymes (CS and HAD) were markedly enhanced in Gr (Table 5). Moreover, PFK activity levels were markedly increased after HypET, but not after ET alone. These results were consistent with previous findings obtained using untrained mice (Suzuki, 2017). Thus, hyperbaric exposure may enhance endurance exercise performance via the upregulation of mitochondrial oxidative capacity and glycolytic capacity in highly trained mice.

Mitochondrial fusion and fission in skeletal muscle are affected by exercise. Mfn1 protein levels decreased during acute endurance exercise and a recovery period up to 24 h, whereas Mfn1 mRNA levels decreased during exercise, but increased at 3 h postexercise and thereafter in rat skeletal muscle (Ding et al., 2010). In contrast, although protein levels of Drp1 did not change, its phosphorylation increased at 1 h during acute endurance exercise and remained elevated until exhaustion, but it returned to pre-exercise levels within 3 h postexercise (Pagano et al., 2014). In the present study, the organs were harvested 48 h after the last exercise bout; therefore, changes in protein expression represented chronic adaptive changes induced by exercise training. In the present study, ET increased Drp1 levels in Gr and DIA (Figure 8). In contrast, SIT alone increased Drp1 levels in Gr and DIA, increased Mfn2 levels in SOL and PL, and increased Drp1 and Mfn2 protein levels in Gw. Thus, ET may preferentially facilitate mitochondrial fission, whereas SIT appears to facilitate fission and fusion in highly trained mice.

In the present study, hyperbaric exposure facilitated Drp1 expression after ET (Figure 8c). The activation of AMPK was identified as a key regulator of mitochondrial fission (Toyama et al., 2016).

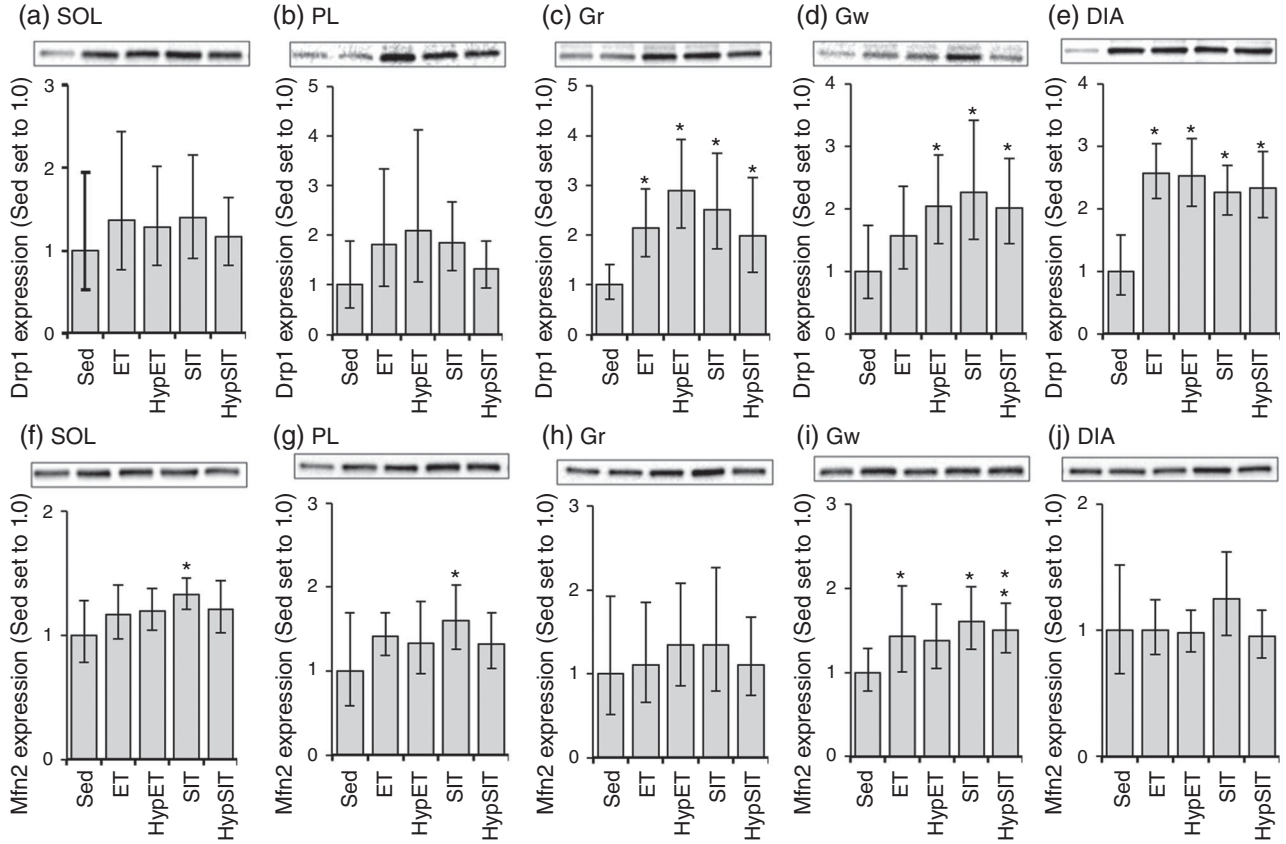

FIGURE 8 Expression of Drp1 (a–e) and Mfn2 (f–j) proteins. Values are represented as means ± SD. *Significantly different from the Sed group at $P < 0.05$ using the Tukey–Kramer multiple comparisons test

Reactive oxygen species (ROS) have been shown to increase AMPK activity (Choi et al., 2001). Hyperbaric exposure of >1 ATA with 100% O_2 was reported to increase ROS production (Thom, 2011). However, ROS was also shown to inhibit AMPK activation in cardiac muscle (Shao et al., 2014). Moreover, enhanced ROS production has not been reported during and/or after mild hyperbaric exposure with 20.9% O_2, as used in the present study. Thus, hyperbaric exposure might facilitate mitochondrial fission by ROS-independent mechanisms. Hyperbaric exposure in the present study facilitated Drp1 expression after ET in Gr (Figure 8c), in which oxidative enzyme levels (CS and HAD) were markedly upregulated by hyperbaric exposure (Table 5). Moreover, enhanced Drp1 levels were not observed after HypSIT. Thus, HypET might facilitate mitochondrial fission by enhancing oxidative metabolism, rather than via the direct effects of hyperbaric exposure.

Consistent with previous findings obtained using untrained mice (Suzuki, 2017), the present study showed that the ET-induced upregulation of HSP70 was facilitated by hyperbaric exposure (Figure 7a–e). After severe exercise (Takemura & Ishihara, 2017) and muscle injury (Oishi et al., 2009), HSP70 plays a crucial role in the recovery of striated muscle. In response to acute exercise and chronic exercise training, HSP70 protein levels were found to increase in rat skeletal muscle (Ogata, Oishi, Higashida, Higuchi, & Muraoka, 2009). Intermittent hyperbaric exposure (3 ATA, 100% O_2, 1 h daily for 5 days consecutively) increased HSP70 mRNA and protein expression levels in mouse neuroblastoma cells (Shyu et al., 2004). The recovery of muscle damage induced by daily exercise may be facilitated by the upregulation of HSP70, resulting in the promotion of muscular adaptation to exercise training.

In DIA, neither ET nor SIT protocols enhanced CS activity values (Table 5). This result is partly supported by previous findings showing that endurance training for 4 weeks did not enhance CS activity values in the rat diaphragm (Suzuki, 2002). In the present study, CS activity values were markedly increased after HypET and HypSIT. Thus, hyperbaric exposure might promote oxidative metabolism in respiratory muscles, thereby enhancing exercise capacity. Chronic intermittent hyperbaric exposure (2.4 ATA with 100% O_2, twice daily for 90 min) for 4 weeks failed to increase oxidative and glycolytic enzyme activity values in rabbit diaphragm (Nelson, Wolf, Hearon, & Li, 1994). Although precise mechanisms could not be determined from the present results, activity-induced adaptation might be facilitated by hyperbaric exposure in respiratory muscles.

4.2 | Effects of hyperbaric exposure on sprint interval training

As described above, HypET markedly enhanced endurance performance. In contrast, HypSIT improved endurance and interval exercise performance, although the absolute work on the last day of the treadmill training was markedly lower with interval exercise [19.1 J (g body weight)$^{-1}$] than with endurance exercise [52.2 J (g body

weight)$^{-1}$]. Thus, HypSIT in the present study appeared to be a beneficial strategy for improving exercise performance in highly trained individuals.

PGC-1α has been shown to stimulate capillary growth induced by exercise training (Lin et al., 2002). The deletion of PGC-1α abolished exercise-induced increases in vascular endothelial growth factor protein levels after 5 weeks of endurance training in mice (Leick et al., 2009). The present results showed that HypSIT facilitated capillary growth, identified by changes in the C:F ratio (Table 4) and PGC-1α expression levels (Figure 6) in glycolytic fibre-rich muscle portions. Thus, facilitated capillary growth might contribute to enhancement of exercise performance by increasing the supply of oxygen and metabolic substrates and promoting a lactate shuttle between organs. In a previous study, 4 weeks of intermittent hyperbaric exposure (1.3 ATA with 20.9% O_2) did not improve capillary growth in skeletal muscles (Suzuki, 2017). Thus, hyperbaric exposure itself might not induce capillary growth, but it might facilitate exercise-induced capillary growth. However, further investigations are needed in order to elucidate the precise mechanisms underlying facilitated capillary growth induced by SIT with chronic intermittent hyperbaric exposure.

Hyperbaric exposure (1.25 ATA, 36% O_2, 3 h per day for 16 weeks) did not increase FCSA in rat soleus muscle (Takemura & Ishihara, 2017). In mouse hindleg muscles, relative muscle mass values did not change after 4 weeks of intermittent hyperbaric exposure (1.3 ATA with 20.9% O_2; Suzuki, 2017). Thus, mild hyperbaric exposure had not been shown to induce muscle hypertrophy. In the present study, relative muscle mass (Table 1) and FCSA (Table 3) were markedly enhanced by HypSIT. As described above, daily exercise volumes in SIT were approximately half those in ET. Thus, HypSIT might be a beneficial strategy for facilitating muscle hypertrophy. Muscle hypertrophy was shown to be induced by multiple anabolic stimuli, including insulin-like growth factor-1 (Egerman & Glass, 2014) and myostatin inhibition (Savage & McPherron, 2010). PGC-1α4, one of the isoforms of PGC-1α, was shown to induce insulin-like growth factor-1 and repress myostatin, thereby increasing muscle mass and strength (Ruas et al., 2012). Unfortunately, the present study did not measure PGC-1α4 expression. The upregulation of PGC-1α by chronic hyperbaric exposure might possibly contribute to facilitated muscle hypertrophy in oxidative and glycolytic fibres, which enhances endurance and interval exercise performance observed after interval training.

In the present study, HypSIT increased the proportion of highly oxidative type I fibres in SOL (Table 2). PGC-1α contributes to the transformation of muscle fibre types by co-activating the calcineurin signalling pathway (Geng et al., 2010). PPARβ was shown to be required for the formation and maintenance of slow oxidative fibres in skeletal muscles by stimulating PGC-1α expression (Schuler et al., 2006). The overexpression of PGC-1α was previously reported to increase the proportion of type I fibres in the gastrocnemius muscle (Lin et al., 2002; Zhang et al., 2017). In the present study, increases in the proportion of type I fibres were observed in SOL only (Table 2). Although the underlying mechanisms have not yet been elucidated in detail, HypSIT might enhance endurance capacity by increasing the proportion of oxidative fibres in highly oxidative muscle.

COMPETING INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

J.S. was involved in the conception and design of the study, analysing the data and preparing the first draft of the manuscript. J.S. revised the drafted manuscript and approved the final version. J.S. agrees to be accountable for all aspects of the work in ensuring that questions relating to the accuracy and integrity of any part of the work are appropriately investigated and resolved. J.S. qualifies for authorship, and all those who qualify are listed.

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