







Leg vascular and skeletal muscle mitochondrial adaptations to aerobic high-intensity exercise training are enhanced in the early postmenopausal phase

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Key points

- Exercise training effectively improves vascular and skeletal muscle function; however, these effects of training may be blunted in postmenopausal women as a result of the loss of oestrogens.
- Accordingly, the capacity to deliver oxygen to the active muscles may also be impaired in postmenopausal women.
- In both premenopausal and recent postmenopausal women, exercise training was shown to improve leg vascular and skeletal muscle mitochondrial function. Interestingly, these effects were more pronounced in postmenopausal women.
- Skeletal muscle oxygen supply and utilization were similar in the two groups of women.
- These findings suggest that the early postmenopausal phase is associated with an enhanced capacity of the leg vasculature and skeletal muscle mitochondria to adapt to exercise training and that the ability to deliver oxygen to match the demand of the active muscles is preserved in the early phase following the menopausal transition.

Abstract Exercise training leads to favourable adaptations within skeletal muscle; however, this effect of exercise training may be blunted in postmenopausal women as a result of the loss of oestrogens. Furthermore, postmenopausal women may have an impaired vascular response to acute exercise. We examined the haemodynamic response to acute exercise in matched pre- and postmenopausal women before and after 12 weeks of aerobic high intensity exercise training. Twenty premenopausal and 16 early postmenopausal (mean \pm SEM: 3.1 \pm 0.5 years after final menstrual period) women only separated by 4 years of age (mean \pm SEM: 50 \pm 0 years *vs.* 54 \pm 1 years) were included. Before training, leg blood flow, O₂ delivery, O₂ uptake and lactate release during knee-extensor exercise were similar in pre- and postmenopausal women. Exercise training reduced ($P < 0.05$) leg blood flow, O₂ delivery, O₂ uptake, lactate release, blood pressure and heart rate during the same absolute workloads in postmenopausal women. These effects were not detected in premenopausal women. Quadriceps muscle protein contents of mitochondrial complex II, III and IV; endothelial nitric oxide synthase (eNOS); cyclooxygenase (COX)-1; COX-2; and oestrogen-related receptor α (ERR α) were increased ($P < 0.05$) with training in postmenopausal women, whereas only the levels of mitochondrial complex V, eNOS and COX-2 were increased ($P < 0.05$) in premenopausal women. These findings demonstrate that vascular and skeletal muscle mitochondrial adaptations to aerobic high intensity exercise training are more pronounced in recent post- compared to premenopausal women, possibly as an effect of enhanced ERR α signalling. Also, the hyperaemic response to acute exercise appears to be preserved in the early postmenopausal phase.

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Abbreviations COX-1, cyclooxygenase-1; eNOS, endothelial nitric oxide synthase; ER, oestrogen receptor; ERE, oestrogen response element; ERR α , oestrogen-related receptor α ; FABF, femoral arterial blood flow; FAP, femoral arterial pressure; FVP, femoral venous pressure; LVC, leg vascular conductance; MAP, mean arterial blood pressure; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator 1 α ; PGI $_2$, prostacyclin; TXA $_2$, thromboxane A $_2$.

Introduction

Exercise training leads to favourable adaptations within skeletal muscle that include improved matching of O $_2$ delivery and O $_2$ demand and increased mitochondrial volume and quality (Blomqvist & Saltin, 1983; Boushel *et al.* 2014; Hellsten & Nyberg, 2015). Because oestrogens modulate a myriad of molecular pathways related to vascular and skeletal muscle function (Phillips *et al.* 1993; Enns & Tiidus, 2010; Menazza & Murphy, 2016), one consequence of oestrogen loss at menopause could be an impaired ability of the active skeletal muscle to attain these adaptations. Accordingly, adaptations in endothelial function in response to low-intensity exercise training have been reported to be reduced in late postmenopausal women compared to age-matched men (Pierce *et al.* 2011; Moreau *et al.* 2013). This impairment appears mainly to be the result of oestrogen loss because oestradiol supplementation concomitant with exercise training can counteract this detrimental consequence of the menopausal transition (Moreau *et al.* 2013). Furthermore, oestrogen treatment has been shown to augment training-induced adaptations in skeletal muscle structure and function in recent postmenopausal women (Taaffe *et al.* 2005).

These beneficial effects of oestrogens are probably mediated via oestrogen receptors (ERs) that are present in both endothelial and skeletal muscle cells and that regulate a number of downstream genes and molecular targets (Enns & Tiidus, 2010; Menazza & Murphy, 2016). The transcriptional activation of ERs by oestrogens is initiated when the receptor binds to specific DNA sequences termed oestrogen response elements (EREs). Interestingly, in addition to oestrogens, muscle contraction can enhance ERE activity in skeletal muscle myotubes (Wiik *et al.* 2009). Because this effect of contractile activity has been found to be independent of functional ERs (Wiik *et al.* 2009), the activation of ERE by contraction may be the result of stimulation of oestrogen-related receptors (ERRs). Activation of ERR by contractile activity is also supported by findings of increased skeletal muscle ERR α mRNA levels following acute exercise in humans (Cartoni *et al.* 2005). It is thus plausible that repeated exercise-induced activation of ERE via activation of ERRs can partly compensate for the loss of oestrogens in postmenopausal women. Moreover, it is possible that intense

aerobic training is more efficient in inducing vascular adaptations (Nyberg *et al.* 2016a,b) and skeletal muscle performance (Seidelin *et al.* 2017) than low to moderate intensity training (Pierce *et al.* 2011; Moreau *et al.* 2013).

In women, acceleration of age-associated declines in vascular and skeletal muscle structure and function are evident after the menopausal transition (Tunstall-Pedoe *et al.* 1994; Taddei *et al.* 1996; Mendelsohn & Karas, 2005; Enns & Tiidus, 2010; Nyberg *et al.* 2016a). The decline in vascular function has been shown to be accompanied by impaired blood flow to skeletal muscle during exercise (Proctor *et al.* 2003; Parker *et al.* 2008). Although the mechanisms underlying the reduced exercise hyperaemia in postmenopausal women remain undisclosed, an impaired vascular response may be attributed to the loss of oestrogens because these strongly influence the regulation of vascular and skeletal muscle function (Phillips *et al.* 1993; Enns & Tiidus, 2010; Menazza & Murphy, 2016). However, the role of oestrogens *vs.* age for the hyperaemic response to exercise has not been possible to discern as a result of a large age-difference (\sim 40 years) between the pre- and postmenopausal women in prior studies (Proctor *et al.* 2003; Parker *et al.* 2008).

In the present study, we tested the hypothesis that, compared to premenopausal women, recent postmenopausal women would display an impaired vascular response to submaximal exercise engaging the knee-extensor muscles. In addition, we hypothesized that aerobic high-intensity training would improve the vascular and metabolic response to knee-extensor exercise to a similar extent in recent postmenopausal women and premenopausal women. Accordingly, we measured haemodynamic variables during submaximal knee-extensor exercise before and after 12 weeks of high-intensity exercise training. To minimize the effects of age-related changes, we investigated pre- and recent postmenopausal women with a minimal (\sim 4 years) difference in age.

Methods

Subjects

Twenty premenopausal women with a mean age of 50 years (range 46–55 years) and 16 postmenopausal women with

Table 1. Subject characteristics

Variable	Premenopausal		Postmenopausal	
	Before training (n = 20)	After training (n = 14)	Before training (n = 16)	After training (n = 16)
Age (years)	50 ± 0	–	54 ± 1 [#]	–
Time from last menstrual cycle (years)	–	–	3.1 ± 0.5	–
Height (m)	1.69 ± 0.01	–	1.67 ± 0.01 [#]	–
Weight (kg)	69.2 ± 1.4	68.0 ± 1.4*	64.4 ± 1.8 [#]	63.1 ± 1.5 ^{#*}
Body mass index (kg m ⁻²)	24.4 ± 0.5	23.9 ± 0.5*	23.1 ± 0.5	22.7 ± 0.4*
Lean body mass (kg)	43.4 ± 0.9	44.2 ± 0.8*	41.6 ± 1.0	42.1 ± 1.0*
Fat percentage (%)	38.5 ± 1.0	36.1 ± 1.1*	36.6 ± 1.1	34.9 ± 1.0*
Waist circumference (cm)	83 ± 1	80 ± 1*	80 ± 2	76 ± 1*
Lean leg mass (kg)	14.3 ± 0.4	14.4 ± 0.4	13.1 ± 0.4 [#]	13.4 ± 0.4*
MAP at rest (mmHg)	90 ± 2	89 ± 3	95 ± 3 [#]	90 ± 3*
Heart rate at rest (beats min ⁻¹)	67 ± 2	65 ± 2	62 ± 2 [#]	58 ± 1 ^{**}
$\dot{V}_{O_{2peak}}$ (mL min ⁻¹)	2106 ± 58	2304 ± 61*	2033 ± 64	2133 ± 65*
$\dot{V}_{O_{2peak}}$ (mL min ⁻¹ kg ⁻¹)	30.6 ± 0.8	34.1 ± 1.1*	31.9 ± 1.3	34.1 ± 1.3*
Peak (RER)	1.28 ± 0.02	1.27 ± 0.04	1.29 ± 0.02	1.26 ± 0.03
Peak workload (W)	199 ± 6	224 ± 6*	194 ± 4	209 ± 5*
Peak heart rate (beats min ⁻¹)	178 ± 2	175 ± 2*	174 ± 2	171 ± 2*
Hematocrit (%)	37.0 ± 0.7	36.4 ± 0.7	36.9 ± 0.6	36.2 ± 0.7
Oestradiol (nmol L ⁻¹)	0.49 ± 0.10	0.52 ± 0.09	0.09 ± 0.01 [#]	0.09 ± 0.01 [#]
Progesterone (nmol L ⁻¹)	6.4 ± 2.7	–	0.9 ± 0.1 [#]	–
FSH (IU L ⁻¹)	12.0 ± 2.8	–	91.5 ± 6.1 [#]	–
LH (IU L ⁻¹)	17.0 ± 2.9	–	34.7 ± 5.0 [#]	–
Testosterone (nmol L ⁻¹)	0.77 ± 0.08	–	0.64 ± 0.07	–
Fasting glucose (mg dL ⁻¹)	5.3 ± 0.1	5.3 ± 0.1	5.2 ± 0.1	5.1 ± 0.1
Total cholesterol (mmol L ⁻¹)	4.9 ± 0.2	4.8 ± 0.1*	5.5 ± 0.2 [#]	5.4 ± 0.1 ^{**}
HDL-cholesterol (mmol L ⁻¹)	1.6 ± 0.1	1.7 ± 0.1	1.9 ± 0.1 [#]	2.0 ± 0.1 [#]
LDL-cholesterol (mmol L ⁻¹)	2.9 ± 0.2	2.8 ± 0.1*	3.2 ± 0.2 [#]	3.1 ± 0.2 ^{**}
Triglycerides (mmol L ⁻¹)	0.83 ± 0.07	0.83 ± 0.07	0.87 ± 0.07	0.81 ± 0.06

Values are the mean ± SE. RER, respiratory exchange ratio; FSH: follicle-stimulating hormone; LH, luteinizing hormone; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Mean arterial pressure (MAP) was measured in the femoral artery. *Significant change ($P < 0.05$) with training; #significantly different ($P < 0.05$) from premenopausal at that time point.

a mean age of 54 years (range 46–57 years) participated in the present study (Table 1). The women were recruited from the Copenhagen area through newspaper advertisement. Eligibility was assessed upon first contact by phone or mail and, second, by evaluation of an online questionnaire and, finally, at a health examination (for details, see below). Recruitment was conducted over four rounds from August 2013 until August 2015 and an almost equal number of pre- and postmenopausal women was recruited in each round to prevent seasonal and investigator-dependent variation. The subjects included in the present study are a part of a larger cohort (Mandrup *et al.* 2016). All subjects were informed of any risks and discomforts associated with the experiments before giving their written, informed consent to participate in the study.

Premenopausal women were all experiencing regular menstrual cycles and were not taking oral contraceptives, whereas postmenopausal women had not experienced

a menstrual cycle during the previous 12 months but were less than 5 years past their final menstrual period (mean ± SE, 3.1 ± 0.5 years). Menopausal status was verified by measurements of hypothalamic and reproductive hormones (Table 1) and none of the postmenopausal women were in hormone replacement therapy. There was no difference between the two groups with respect to previous exposure to reproductive hormones and parity. All subjects were habitually inactive (less than 2 h of moderate intensity exercise per week), although many used a bicycle for daily transportation. All women had normal resting ECG, were non-smokers, and none of the subjects in either group had been diagnosed with hypertension, cardiovascular disease, renal dysfunction, insulin resistance, diabetes or hypercholesterolaemia. To ensure that all women were normotensive at inclusion, mean arterial blood pressure (MAP) was measured seven consecutive times by an automatic upper arm blood pressure monitor (M7;

Omron, Vernon Hills, IL, USA) after at least 15 min of rest in the supine position (premenopausal: $112 \pm 3/72 \pm 2$ mmHg; postmenopausal: $114 \pm 4/73 \pm 2$ mmHg).

The data presented are part of a larger study on the effects of exercise training on women in the menopausal transition funded via the University of Copenhagen Excellence Programme for Interdisciplinary Research and the data presented in Tables 1 and 2 have been reported previously (Mandrup *et al.* 2016; Nyberg *et al.* 2016a). The study was approved by the Ethics Committee of Copenhagen and Frederiksberg communities Region H (H-1-2012-150) and conducted in accordance with the guidelines of the *Declaration of Helsinki*. The study was registered at ClinicalTrials.gov (NCT02135575).

Experimental design

The experimental days were performed before and after a 12-week training period where the subjects participated in supervised exercise training. Experimental testing in premenopausal women was performed in the mid-follicular phase of their ovarian cycle.

Exercise training

Subjects performed exercise on a cycling ergometer for 1 h three times per week of which two of these occasions were supervised by instructors from the research group and one was supervised by instructors from the local gym. A heart rate monitor was worn during all training sessions (TEAM2 Wearlink+; Polar, Kempele, Finland). Exercise intensity during the training sessions is presented in Table 2.

Before and after the training intervention, peak $\dot{V}O_{2\text{peak}}$ was determined (Oxycon Pro; Intra-medical, Gentofte, Denmark) using of an incremental exercise test on a cycling ergometer. Briefly, after a standardized 10 min warm-up, the test was initiated at 50 W and the workload was increased by 25 W every 1 min until volitional fatigue.

Experimental day 1

Subjects refrained from caffeine, alcohol, and exercise for 24 h before arriving at the laboratory after eating breakfast. After local anaesthesia (lidocaine, 20 mg ml⁻¹; Astra Zeneca, Copenhagen, Denmark), catheters (20 G; Arrow International, Reading, PA, USA) were placed in the femoral artery and vein of the experimental leg and in the femoral artery of the non-experimental leg. After ~60 min of rest, subjects performed 5 min of one-leg knee-extensor exercise (Andersen & Saltin, 1985) at 10 W followed by 5 min at 20 W in an upright seated position. Subjects were instructed to maintain a kicking frequency of 60 rpm and a passive return of the leg to

Table 2. Training

Group	Training sessions	Average time per session (min:s)	Total training time (% of maximum heart rate)						
			< 61%	61–70%	71–80%	81–85%	86–90%	91–95%	96–100%
Premenopausal (n = 20)	36.3 ± 1.6	53:10 ± 00:31	2.3 ± 2.0	7.9 ± 4.1	25.5 ± 6.7	22.4 ± 4.7	26.9 ± 7.9	13.4 ± 7.4	1.5 ± 0.4
Postmenopausal (n = 16)	34.9 ± 1.3	51:27 ± 01:11	1.8 ± 0.5	7.6 ± 1.2	21.7 ± 2.2	22.1 ± 1.3	30.1 ± 3.1	15.2 ± 2.2	1.4 ± 0.4

Values are means ± SE.

minimize involvement of the hamstring muscles. The workloads were chosen to reflect light/moderate (10 W) and moderate/high-intensity (20 W) and to ensure that all women could perform the exercise for 5 min at each workload. Blood flow, blood pressures and arterial and venous blood samples (~2 ml) were obtained after 4.5 min at each workload.

Experimental day 2

Subjects refrained from caffeine, alcohol and exercise for 24 h before arriving at the laboratory in a fasted state. A blood sample from the antecubital vein and a biopsy from the musculus vastus lateralis (obtained through an incision made in the skin under local anaesthesia; lidocaine, 20 mg mL⁻¹; Astra Zeneca) were then collected. This experimental day was separated by at least 48 h from experimental day 1.

Measurement and analysis

Body composition (fasted state) was assessed by dual X-ray absorptiometry scanning (Lunar iDXA; GE Healthcare, Little Chalfont, UK) by the same investigator blinded for menopausal status. Whole blood samples obtained from the antecubital vein (experimental day 2) were analysed at the clinical biochemical unit at Rigshospitalet (University Hospital of Copenhagen) via an automatic analyser using a turbidimetric immunoassay (Modular/COBAS P-Module; Roche Diagnostics, Basel, Switzerland); C-reactive protein, oestradiol, progesterone and testosterone were analysed via a competitive electrochemiluminescence immunoassay (Modular/COBAS E-Module); and follicle-stimulating hormone and luteinizing hormone were analysed via a sandwich electrochemiluminescence immunoassay (Modular/COBAS E-Module). Plasma high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, total cholesterol and triglyceride were measured using enzymatic absorption photometry (Modular/COBAS C-Module). Glucose was determined by amperometric metabolite electrode measurement (ABL 837; Radiometer, Copenhagen, Denmark).

Femoral arterial blood flow (FABF) was measured with ultrasound Doppler (Vivid E9; GE Healthcare, Brøndby, Denmark) equipped with a linear probe operating at an imaging frequency of 8.0 MHz and a Doppler frequency of 3.1 MHz as described previously (Nyberg *et al.* 2014a). Intra-arterial and intra-venous pressures were monitored with transducers (Pressure Monitoring Set; Edwards Lifesciences, Irvine, CA, USA) positioned at the level of the tip of the catheter. Blood gases, haemoglobin, pH and lactate were measured using an ABL800 FLEX analyser (Radiometer). Leg vascular conductance (LVC) was calculated as FABF/[mean femoral arterial pressure (FAP) – mean

femoral venous pressure (FVP)]. Leg O₂ uptake was calculated as the arterio-venous O₂ difference multiplied by FABF; leg O₂ delivery as FABF multiplied by arterial O₂ content; and leg lactate release as the venous–arterial lactate difference multiplied by FABF.

In the muscle samples, protein expression was determined by western blotting. Freeze-dried muscle tissue samples obtained at rest were homogenized in a fresh batch of buffer [10% glycerol, 20 mM sodium-pyrophosphate, 150 mM NaCl, 50 mM Hepes, 1% Nonidet P-40 (Shell Chemicals, Hague, The Netherlands), 20 mM β-glycerophosphate, 10 mM NaF, 2 mM phenylmethane sulphonyl fluoride, 1 mM each EDTA and EGTA, 10 μg mL⁻¹ each aprotinin and leupeptin and 3 mM benzamidin] two times for 30 s (Qiagen Tissuelyser II; Retsch, Haan, Germany). After rotation end over end for 1 h, the samples were centrifuged for 30 min at 17 500 g at 4°C and the lysate was collected as the supernatant. Protein concentrations were determined in the lysates (assayed in triplicate) using BSA standards (Pierce Reagents, Rockford, IL, USA). The lysates were diluted to appropriate protein concentrations (2 μg μl⁻¹) in a ×6 sample buffer (0.5 M Tris-base, dithiothreitol, SDS, glycerol and bromphenol blue) and an equal amount of total protein was loaded for each sample in different wells on precast gels (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of total protein were loaded for each sample in accordance with the antibody optimization: ERRα (5 μg), total OXPHOS (1 μg), endothelial nitric oxide synthase (eNOS) (6 μg), cyclooxygenase (COX)-1 (5 μg), COX-2 (8 μg), prostacyclin (PGI₂) synthase (8 μg) and thromboxane A₂ (TXA₂) synthase (4 μg). Samples from each group were distributed evenly across the gel and the sample from before training was placed adjacent to the sample after training for each subject. In addition, a human standard sample was also loaded to control for differences over the gel/membrane. After gel electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane, which was incubated with primary antibody overnight and then washed for 5 min in Tris-buffered saline with Tween-20 before incubation with secondary antibody for 1 h.

Antibodies used were: ERRα (dilution 1:1000; 2% non-fat milk; Abcam, Cambridge, UK), total OXPHOS (dilution 1:1500; 5% non-fat milk; Abcam), eNOS (dilution 1:500; 2% non-fat milk; BD Biosciences, Franklin Lakes, NJ, USA), COX-1 (dilution 1:600; 3% BSA; Abcam), COX-2 (dilution 1:500; 2% non-fat milk; Abcam), PGI₂ synthase (dilution 1:200; 2% non-fat milk; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and TXA₂ synthase (dilution 1:500; 2% non-fat milk; Abcam). The secondary HRP-conjugated antibody (Dako, Glostrup, Denmark) was diluted 1:5000 in 2/5% non-fat milk or 3% BSA, depending on the primary antibody. The membrane staining was visualized by incubation with

a chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) and the image was digitalized on a ChemiDoc MP system (Bio-Rad). All proteins were expressed in arbitrary units.

Statistical analysis

A linear mixed-model approach was used to investigate differences within and between groups. Fixed factors were 'group' (premenopausal, postmenopausal), 'time' (before training, after training) and 'intensity' (10 W, 20 W). Subjects were specified as a repeated factor and identifier of random variation. Homogeneity of covariance and normal distribution were confirmed through residual and Q-Q plots. Pairwise differences were identified using Tukey's honestly significant difference *post hoc* procedure. Pearson correlation analysis was used to determine relations of interest. As a result of technical difficulties, haemodynamic data obtained after training included 14 of the 20 premenopausal women (also clarified in each figure legend). The number of subjects was selected on the basis of detecting differences in blood flow to the exercising leg between the two groups before training and within each group with training. Statistical analyses were performed with R, version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria) through the interface R studio (R Foundation for Statistical Computing). Data are reported as the mean \pm SE. $P < 0.05$ was considered statistically significant.

Results

Subject characteristics

Before the training period, there was no difference in $\dot{V}_{O_{2peak}}$ between pre- and postmenopausal women (Table 1). Exercise training increased ($P < 0.05$) $\dot{V}_{O_{2peak}}$ by $9.9 \pm 2.4\%$ and $5.4 \pm 2.1\%$ in the pre- and postmenopausal women. Before training, MAP at rest was higher ($P < 0.05$) in postmenopausal women compared to premenopausal women, although this difference in MAP was not detected after training as a result of a training-induced lowering ($P < 0.05$) of MAP in the postmenopausal group. Heart rate at rest was lower ($P < 0.05$) in postmenopausal women before and after training. Exercise training lowered heart rate at rest in postmenopausal women, whereas there was a non-significant reduction in premenopausal women ($P = 0.053$).

Vascular and metabolic responses to knee-extensor exercise

FABF and leg O_2 delivery. During knee-extensor exercise, FABF and leg O_2 delivery were similar in the pre- and postmenopausal women before training (Fig. 1A and B). In the postmenopausal group, exercise training reduced

($P < 0.05$) FABF ($-10.8 \pm 3.6\%$ and $-6.4 \pm 2.8\%$; 10 and 20 W) and O_2 delivery ($-12.6 \pm 3.9\%$ and $-13.8 \pm 3.5\%$) and FABF and O_2 delivery were lower ($P < 0.05$) in postmenopausal compared to premenopausal women after training.

Leg arterio-venous O_2 difference and O_2 uptake. Leg arterio-venous O_2 difference was similar in both groups of women and was not changed with training (Fig. 1D). Leg O_2 uptake was lower ($-9.2 \pm 3.4\%$ and $-10.3 \pm 3.7\%$; 10 and 20 W; $P < 0.05$) after training in postmenopausal women and leg O_2 uptake was lower ($P < 0.05$) in postmenopausal compared to premenopausal women after training (Fig. 1F).

Difference between leg O_2 delivery and O_2 uptake. Before training, the difference between leg O_2 delivery and leg O_2 uptake was not different between the pre- and postmenopausal women (Fig. 2A). The difference between leg O_2 delivery and leg O_2 uptake was lower ($P < 0.05$) after training in both the pre- ($-6.4 \pm 9.3\%$ and $-4.6 \pm 5.8\%$; 10 and 20 W) and postmenopausal group ($-18.3 \pm 5.3\%$ and $-19.0 \pm 5.2\%$). The difference between leg O_2 delivery and leg O_2 uptake was reduced to a larger extent ($P < 0.05$) at 20 W in the postmenopausal compared to the premenopausal group (-17 ± 12 vs. -46 ± 11 mL O_2 min^{-1} , pre- and postmenopausal). There was a linear relationship ($r^2 = 0.97$, $P < 0.05$) between leg O_2 uptake and leg O_2 delivery irrespective of training status (Fig. 2B).

Leg venous-arterial lactate difference and lactate release.

Before training, leg venous-arterial lactate difference and lactate release were not different between the two groups (Fig. 1C). Exercise training reduced ($P < 0.05$) leg venous-arterial lactate difference in pre- (-0.2 ± 0.1 and -0.3 ± 0.1 mmol L^{-1} , 10 and 20 W) and postmenopausal (-0.3 ± 0.1 and -0.2 ± 0.1 mmol L^{-1}) women, whereas lactate release was only reduced ($P < 0.05$) in postmenopausal women (-0.8 ± 0.2 and -0.9 ± 0.4 mmol min^{-1}) (Fig. 1C and E).

Mean femoral arterial blood pressure. FAP was higher ($P < 0.05$) in postmenopausal women compared to premenopausal women before but not after training because training reduced ($P < 0.05$) FAP in the postmenopausal group (-12.0 ± 2.9 and -10.5 ± 2.9 mmHg; 10 and 20 W) (Fig. 3A). LVC was not different between groups before training (Fig. 3B). Although changes in LVC with training did not reach statistical difference in any of the groups, LVC was lower ($P < 0.05$) in postmenopausal women compared to premenopausal women after training. Before training, a negative correlation was detected between LVC during 10 W and follicle-stimulating hormone when accounting for all women ($r^2 = 0.16$, $P = 0.024$).

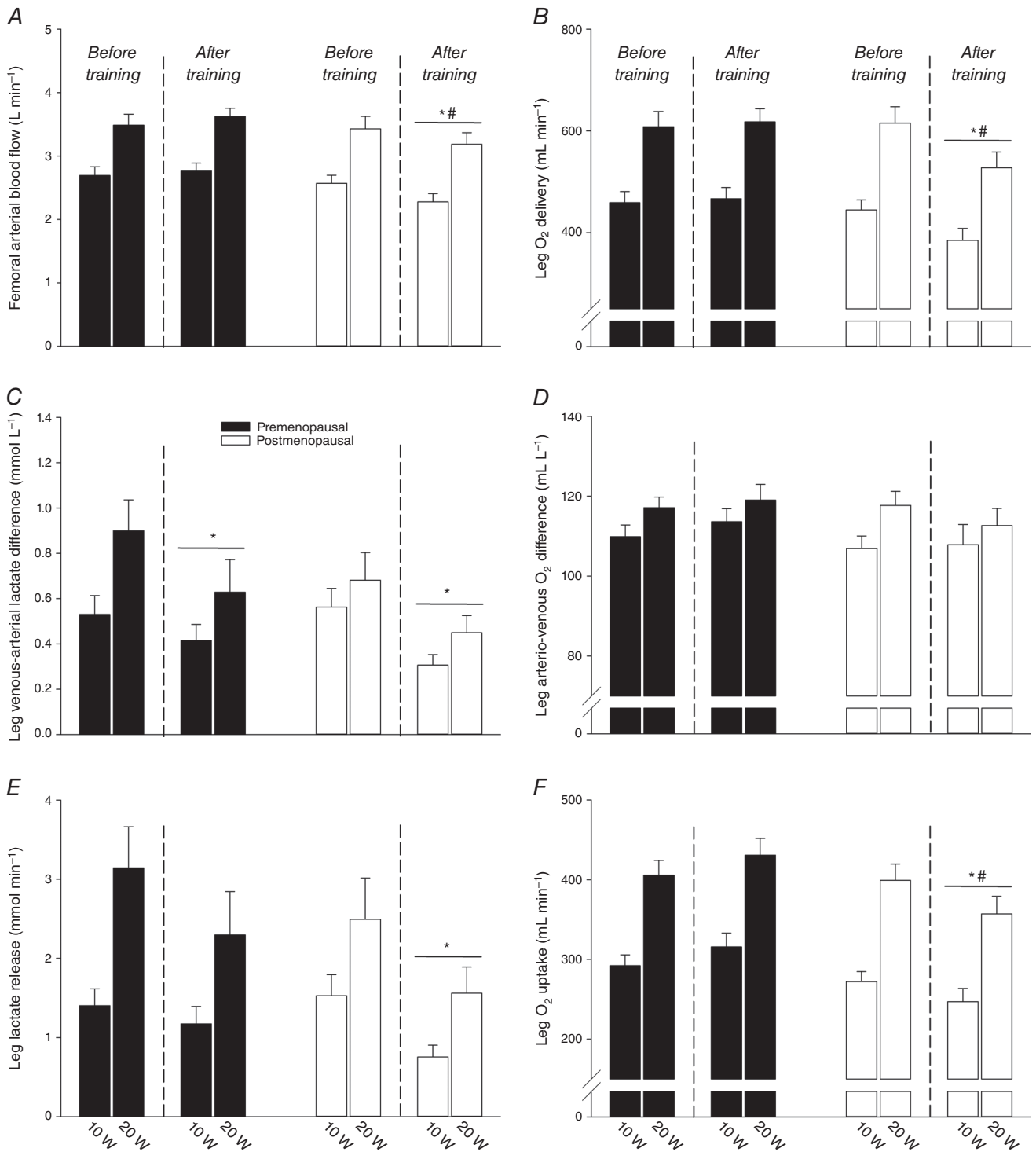


Figure 1. Leg haemodynamics during exercise

Femoral arterial blood flow (A), leg O₂ delivery (B), leg venous-arterial-lactate difference (C), leg arterio-venous O₂ difference (D), leg lactate release (E) and leg O₂ uptake (F) in pre- ($n = 14-20$) and postmenopausal ($n = 16$) women during knee-extensor exercise performed at 10 and 20 W before and after training. *Significantly different ($P < 0.05$) from before training; #significantly different ($P < 0.05$) from premenopausal at that time point.

Blood pO_2 , O_2 content, pH, O_2 saturation, haemoglobin, heart rate and FVP are presented in Table 3.

Skeletal muscle protein content

In muscle homogenate, the content of ERR α was similar in the two groups before training (Fig. 4). In the postmenopausal group but not in the premenopausal group, the level of ERR α increased ($\sim 62\%$, $P < 0.05$) with training so that the protein content of ERR α was higher ($P < 0.05$) in the post- compared to premenopausal group after training.

The protein level of complex I–V (OXPHOS) was similar in the two groups before training (Fig. 5). In premenopausal women, the protein content of complex V was higher ($\sim 28\%$, $P < 0.05$) after training, whereas

the content of complex II ($\sim 50\%$), III ($\sim 33\%$) and IV ($\sim 33\%$) was higher ($P < 0.05$) in the postmenopausal group after training. The level of complex II and III was higher ($P < 0.05$) in the post- compared to premenopausal women after training. A negative correlation ($r^2 = 0.35$, $P = 0.047$) was detected between the training-induced change in complex IV and lactate release during 10 W in postmenopausal women

Before training, the protein content of eNOS, COX-1, COX-2, PGI $_2$ synthase and TXA $_2$ synthase was similar in the two groups of women (Fig. 6). In the premenopausal women, the level of eNOS ($\sim 29\%$) and COX-2 ($\sim 60\%$) was higher ($P < 0.05$) after training. In the postmenopausal women, the level of eNOS ($\sim 49\%$), COX-1 ($\sim 55\%$) and COX-2 ($\sim 105\%$) was increased ($P < 0.05$) with training. These effects of

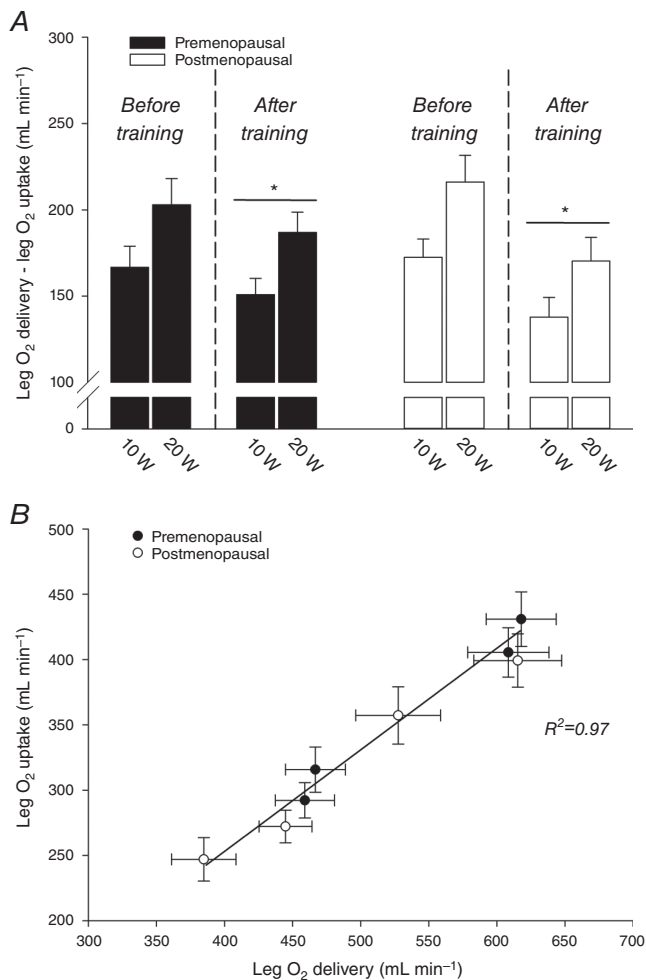


Figure 2. Matching of O_2 delivery and O_2 utilization during exercise

Difference between leg O_2 delivery and leg O_2 uptake (A) and the association between leg O_2 delivery and leg O_2 uptake (B) in pre- ($n = 14$ – 20) and postmenopausal ($n = 16$) women during knee-extensor exercise performed at 10 and 20 W before and after training. *Significantly different ($P < 0.05$) from before training.

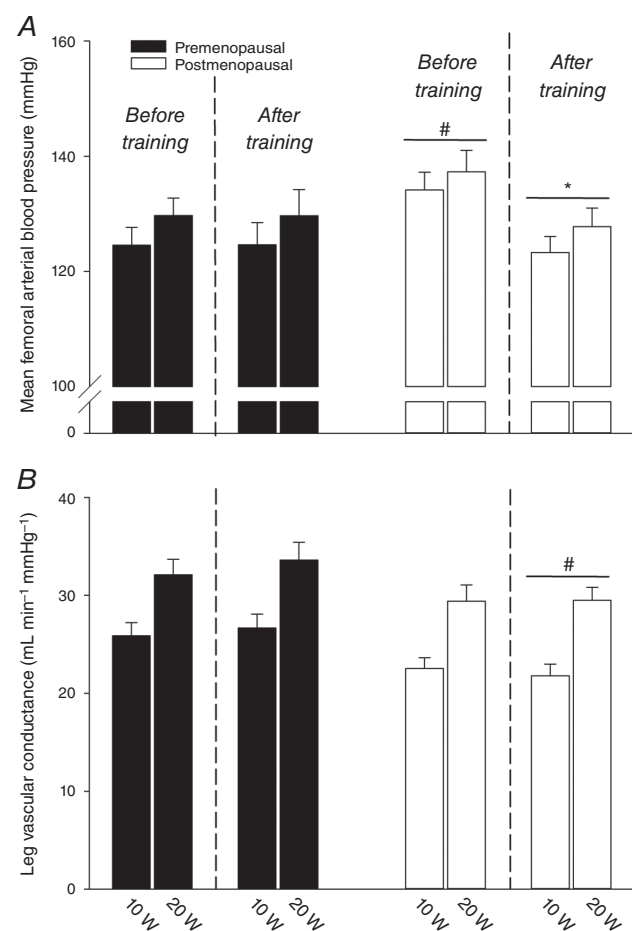


Figure 3. Blood pressure and vascular conductance during exercise

Mean femoral arterial blood pressure (A) and leg vascular conductance (B) in pre- ($n = 14$ – 20) and postmenopausal ($n = 16$) women during knee-extensor exercise performed at 10 and 20 W before and after training. *Significantly different ($P < 0.05$) from before training; #significantly different ($P < 0.05$) from premenopausal at that time point.

Table 3. Blood and haemodynamic variables during knee-extensor exercise

Blood variable	Premenopausal						Postmenopausal						
	Before training (n = 20)			After training (n = 14)			Before training (n = 16)			After training (n = 16)			
	10 W	20 W	20 W	10 W	10 W	20 W	10 W	10 W	20 W	10 W	10 W	20 W	
P_O₂ (mmHg)													
Arterial	99 ± 2	102 ± 2	102 ± 2	102 ± 2	106 ± 2	106 ± 2	102 ± 2	108 ± 3	103 ± 2	105 ± 2			
Venous	24 ± 1	24 ± 1	23 ± 1*	23 ± 1*	23 ± 1*	23 ± 1*	25 ± 1	25 ± 1	25 ± 1*	23 ± 1*			
Haemoglobin (mmol L⁻¹)													
Arterial	8.0 ± 0.1	8.1 ± 0.1	7.8 ± 0.2	7.8 ± 0.2	7.9 ± 0.2	7.9 ± 0.2	8.1 ± 0.2	8.4 ± 0.2	7.7 ± 0.2*	7.7 ± 0.2*			
Venous	7.9 ± 0.1	8.1 ± 0.1	7.8 ± 0.2	7.8 ± 0.2	7.9 ± 0.2	7.9 ± 0.2	8.0 ± 0.1	8.1 ± 0.2	7.7 ± 0.2*	7.7 ± 0.2*			
O₂ saturation (%)													
Arterial	97.8 ± 0.1	98.0 ± 0.2	97.9 ± 0.2*	97.9 ± 0.2*	98.6 ± 0.5*	98.6 ± 0.5*	98.0 ± 0.2	98.1 ± 0.2	97.8 ± 0.1	98.0 ± 0.1			
Venous	35.8 ± 1.4	32.5 ± 1.5	31.9 ± 1.5*	31.9 ± 1.5*	29.9 ± 1.5*	29.9 ± 1.5*	38.7 ± 1.7	35.6 ± 1.7	35.8 ± 2.0*	31.6 ± 1.4*			
O₂ content (mL L⁻¹)													
Arterial	172 ± 3	175 ± 3	168 ± 4	168 ± 4	171 ± 4	171 ± 4	174 ± 3	181 ± 3	166 ± 3*	165 ± 3*			
Venous	62 ± 2	58 ± 3	55 ± 3*	55 ± 3*	52 ± 3*	52 ± 3*	67 ± 3	63 ± 3	59 ± 3*	53 ± 2*			
pH													
Arterial	7.405 ± 0.003	7.410 ± 0.004	7.413 ± 0.005	7.413 ± 0.005	7.417 ± 0.008	7.417 ± 0.008	7.403 ± 0.005	7.393 ± 0.009	7.407 ± 0.004*	7.412 ± 0.004*			
Venous	7.319 ± 0.006	7.295 ± 0.008	7.329 ± 0.008*	7.329 ± 0.008*	7.314 ± 0.01*	7.314 ± 0.01*	7.310 ± 0.007	7.280 ± 0.011	7.332 ± 0.005*	7.318 ± 0.004*			
Lactate (mmol L⁻¹)													
Arterial	1.2 ± 0.1	1.9 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.6 ± 0.3	1.6 ± 0.3	1.2 ± 0.1	2.3 ± 0.3	0.8 ± 0.1*	1.3 ± 0.2*			
Venous	1.7 ± 0.2	2.8 ± 0.3	1.5 ± 0.2	1.5 ± 0.2	2.3 ± 0.4	2.3 ± 0.4	1.8 ± 0.2	2.9 ± 0.3	1.2 ± 0.1*	1.8 ± 0.3*			
Heart rate (beats min⁻¹)													
Arterial	92 ± 2	104 ± 3	89 ± 3	89 ± 3	101 ± 5	101 ± 5	86 ± 3	102 ± 3	74 ± 2**	86 ± 4**			
FVP (mL min⁻¹ mmHg⁻¹)													
Arterial	19.7 ± 0.8	20.4 ± 1.1	19.1 ± 0.8	19.1 ± 0.8	19.8 ± 0.9	19.8 ± 0.9	19.6 ± 1.4	20.0 ± 1.6	18.5 ± 1.2	20.1 ± 1.5			

Data are the mean ± SE. # Significantly different (P < 0.05) from premenopausal (main effect). *Significant change (P < 0.05) with training (main effect).

training resulted in a higher ($P < 0.05$) level of eNOS and COX-1, although there were similar levels of COX-2 in the post- compared to premenopausal women after training. The protein content of PGI₂ synthase and TXA₂ synthase was not changed as a result of training.

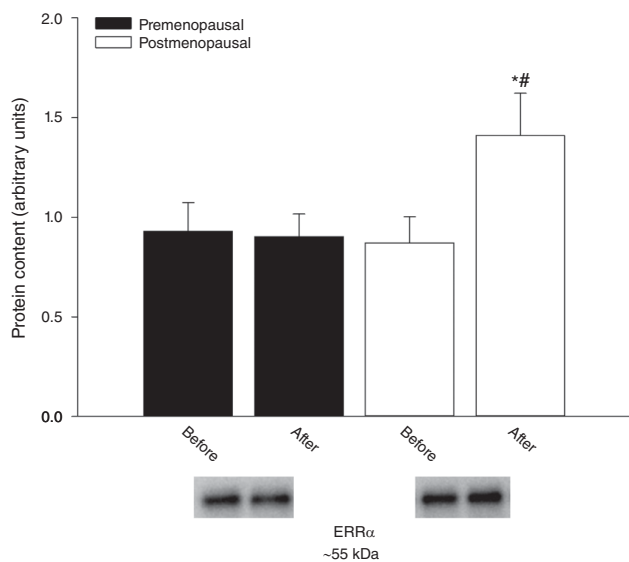


Figure 4. Protein content of ERR α in vastus lateralis muscle

Protein content of ERR α in vastus lateralis muscle before and after training in pre- ($n = 15$) and postmenopausal ($n = 13$) women. *Significantly different ($P < 0.05$) from before training; #significantly different ($P < 0.05$) from premenopausal at that time point.

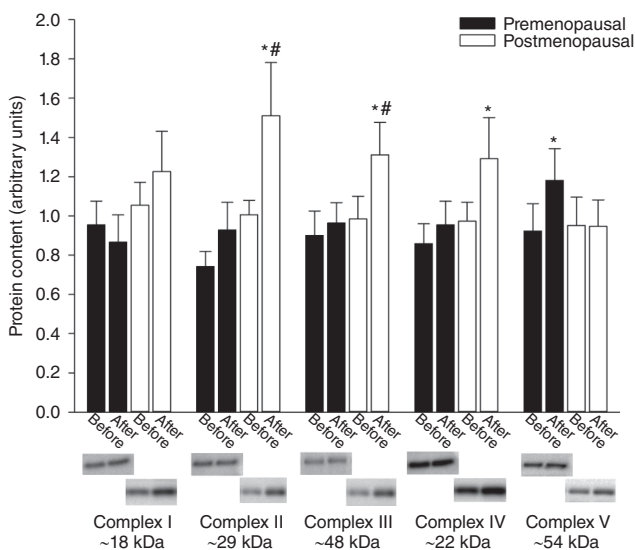


Figure 5. Protein content of mitochondrial complex I–V in vastus lateralis muscle

Protein content of mitochondrial complex I–V in vastus lateralis muscle before and after training in pre- ($n = 14$ – 17) and postmenopausal ($n = 12$ – 14) women. *Significantly different ($P < 0.05$) from before training; #significantly different ($P < 0.05$) from premenopausal at that time point.

Discussion

There were two primary findings of the present study. (i) A period of aerobic high-intensity exercise training induced leg vascular and skeletal muscle mitochondrial adaptations in both pre- and postmenopausal women; however, these effects of training were most pronounced in postmenopausal women. (ii) Before initiation of training, pre- and postmenopausal women displayed a similar vascular and metabolic response to acute exercise engaging the knee-extensor muscles.

Role of menopausal status in the adaptive response to exercise training

Salient responses to aerobic exercise training include augmented maximal skeletal muscle blood flow and mitochondrial oxidative capacity. Moreover, exercise training leads to a more precise matching of O₂ delivery and oxidative metabolism during submaximal exercise through optimization of microvascular blood flow distribution in the muscle (Saltin *et al.* 1976; Kalliokoski *et al.* 2001; Hellsten & Nyberg, 2015). In the present study, the difference between O₂ delivery and O₂ uptake was reduced in the pre- and postmenopausal women, indicating that training improved the matching of blood flow and oxidative metabolism in both groups. This effect of training was evident in both groups of women and, interestingly, the effect was more pronounced in the postmenopausal group during the highest workload.

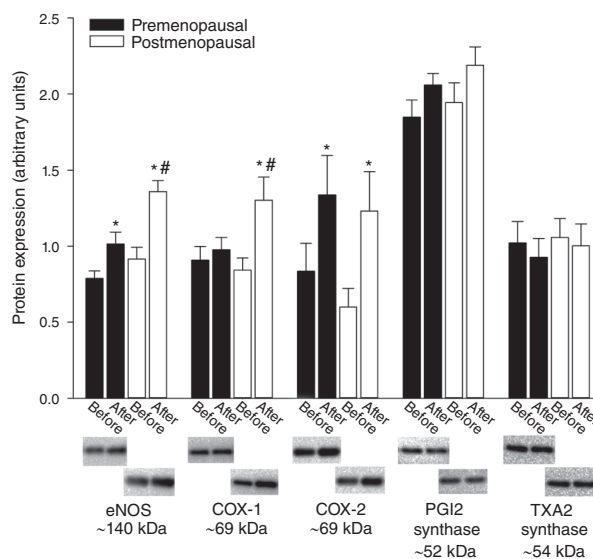


Figure 6. Protein content of eNOS, COX-1, COX-2, PGI₂ synthase and TXA₂ synthase in vastus lateralis muscle

Protein content of eNOS, COX-1, COX-2, PGI₂ synthase and TXA₂ synthase in vastus lateralis muscle before and after training in pre- ($n = 16$ – 17) and postmenopausal ($n = 13$ – 14) women. *Significantly different ($P < 0.05$) from before training; #significantly different ($P < 0.05$) from premenopausal at that time point.

Along these lines, lactate release from the exercising leg was reduced after training only in postmenopausal women, indicating that, in this group, the contribution of energy from aerobic sources was enhanced. This effect of training is in agreement with the marked increase in mitochondrial content in postmenopausal women because a lower lactate production following exercise training has been shown to be a result of increased mitochondrial volume (Holloszy, 1967; Boushel *et al.* 2014). One mechanism underlying the more prominent response to exercise training in postmenopausal women could be an enhanced signalling through the ERR α pathway. ERR α has been shown to be an effector of peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) and a regulator of genes involved in oxidative phosphorylation and mitochondrial biogenesis (Schreiber *et al.* 2004). In agreement with this proposed mechanism of action, the skeletal muscle protein content of ERR α was found to increase in postmenopausal women only. Because oestrogens readily interact with ERR α (Vanacker *et al.* 1999), this pathway might not be further stimulated by muscle contraction in the premenopausal state. In this setting, a loss of oestrogens may lead to an enhanced contraction-induced signalling through the ERR α pathway. This altered signalling in response to contraction may require sufficiently high exercise intensity because intense exercise (Nyberg *et al.* 2016a,b) but not low- to moderate-intensity exercise (Pierce *et al.* 2011; Moreau *et al.* 2013) is associated with improved vascular function in postmenopausal women. However, it is also plausible that the proposed contraction-activated ERR α pathway is no longer as readily stimulated many years after menopause because the latter studies used late postmenopausal women (Pierce *et al.* 2011; Moreau *et al.* 2013). The putative role of the PGC-1 α - ERR α signalling pathway in mitochondrial adaptations to exercise training in postmenopausal women deserves further investigation.

An improved matching of microvascular blood flow and oxidative metabolism in contracting skeletal muscle could be explained, at least in part, by the enhanced function of the NO and prostanoid systems because these systems have been shown to be essential for exercise hyperaemia (Radegran & Saltin, 1999; Mortensen *et al.* 2007) and to be improved with exercise training (Nyberg *et al.* 2012). In agreement, skeletal muscle eNOS and COX-2 content was increased in both groups, indicating that both the capacity and the function of these systems were improved. Also, the more pronounced increase in content of eNOS with training in postmenopausal women is in agreement with an enhanced ERR α signalling in this group of women because ERR α is involved in the regulation of eNOS mRNA and protein expression (Sumi & Ignarro, 2003).

Despite substantial peripheral training adaptations in postmenopausal women, including increased skeletal muscle mitochondrial content, no increase in leg

arterio-venous O₂ difference was observed. Exercise training has been shown to increase O₂ extraction and reduce blood flow during submaximal exercise (Kiens *et al.* 1993; Kalliokoski *et al.* 2001; Hellsten & Nyberg, 2015), although this is not a consistent finding (Beere *et al.* 1999; Mortensen *et al.* 2012; Gifford *et al.* 2016). The reason for the lack of change is not clear, although it may be that more enhanced structural and functional adaptations than those observed in the present study are needed for a greater O₂ extraction during submaximal exercise.

Skeletal muscle blood flow and O₂ delivery are closely matched to the O₂ demand of the contracting muscles during one-leg knee extensor exercise and submaximal cycling (Andersen & Saltin, 1985; Gonzalez-Alonso *et al.*, 2002, 2008; Mortensen *et al.* 2008). Given the unaltered leg arterio-venous O₂ difference, the lower blood flow in postmenopausal women was likely a consequence of reduced O₂ demand of the exercising leg. It is well established that exercise training can reduce the metabolic cost of locomotion as indicated by a lower steady-state O₂ consumption at a given submaximal intensity following a period of training (Bransford & Howley, 1977; Proctor *et al.* 2001; Nyberg *et al.* 2016b). Such an effect of training was also observed in the present study because leg O₂ uptake during exercise was lower in postmenopausal women after training. Although more efficient mitochondria (i.e. an improved P/O ratio) is an attractive candidate for the lower O₂ uptake, the finding of a similar P/O ratio in sedentary and well trained subjects (Mogensen *et al.* 2006) suggests that other mechanisms may be more important. Both *in vitro* (Barclay, 1996) and *in vivo* studies (Krustrup *et al.* 2008) have provided evidence showing that type I fibres are more economical than type II fibres and enhanced PGC-1 α signalling has also been shown to induce fibre type switching from fast, glycolytic fibres towards slow oxidative fibres (Lin *et al.* 2002). Hence, an enhanced PGC-1 α signalling via ERR α in postmenopausal women may have induced not only an increased mitochondrial capacity, but also a fibre type switch towards more oxidative fibres that reduced the metabolic cost of contraction. In addition, the reduced oxidative metabolism may also reflect an optimized recruitment of accessory and/or active muscle fibres (higher dependence on type I fibres), which would reduce the overall O₂ demand (Krustrup *et al.* 2008), although the mechanisms by which menopausal status affects such adaptations are unclear.

The classical view of $\dot{V}_{O_{2max}}$ is that maximal rates of O₂ utilization in skeletal muscle are limited under most circumstances by the ability of the heart to deliver O₂ to the mitochondria (Saltin & Strange, 1992). This is probably true in physically active subjects (Levine, 2008; Hellsten & Nyberg, 2015); however, evidence for $\dot{V}_{O_{2max}}$ being limited by the capacity of the mitochondria to utilize O₂ in sedentary subjects has been reported (Jacobs *et al.*

2013; Gifford *et al.* 2016). Hence, the rate-limiting step in the O₂ cascade may also be shifted in postmenopausal women because the action of skeletal muscle ER α has been demonstrated to be critical for mitochondrial function (Ribas *et al.* 2016). Accordingly, although the difference in the training-induced change in $\dot{V}_{O_2\text{peak}}$ and peak workload between the two groups did not reach statistical significance, the numerically greater change in the premenopausal group is intriguing. Because this observation was paralleled by enhanced peripheral adaptations in postmenopausal women (although the functional adaptations were detected during knee-extensor exercise where muscle blood flow is not expected to be limited by cardiac output), it may be speculated that the increase in $\dot{V}_{O_2\text{peak}}$ in this group was mediated by improved mitochondrial capacity, whereas the increase in premenopausal women was mediated by a greater peak cardiac output.

Another observation in the present study was a training-induced reduction in heart rate and blood pressure during exercise observed only in postmenopausal women. These adaptations are in agreement with previous findings (Saltin *et al.* 1976; Mortensen *et al.* 2013) and the lower heart rate observed in the present study probably reflects an increased stroke volume in addition to lower cardiac output as a result of the reduced leg blood flow (Hellsten & Nyberg, 2015). Local factors within the skeletal muscle probably play an important role in this altered haemodynamic response to acute exercise with training. Accordingly, afferent fibres located within skeletal muscles respond to mechanical distortion (group III) and changes in the chemical environment (group IV) (e.g. interstitial pH, ATP and lactate), which contributes to the increase in sympathetic nervous activity and consequently heart rate and blood pressure during exercise (i.e. the exercise pressor reflex) (Mitchell *et al.* 1983; Fadel, 2015). The observed reduction in lactate release with training probably reflects lower interstitial levels and could partly explain the lower heart rate and blood pressure during exercise observed only in the postmenopausal women after training.

Longitudinal and cross-sectional data have provided compelling evidence that exercise training leads to an increase in total haemoglobin mass, whereas hematocrit either remains unaltered or is slightly reduced as a result of plasma expansion (Hellsten & Nyberg, 2015). The reason that the Hb concentration was only reduced in the postmenopausal women after the training is unclear, although it may be related to a greater increase in plasma volume or potentially a reduced ability to synthesize erythrocytes in the premenopausal group.

Role of menopausal status in the regulation of exercise hyperaemia

There is accumulating evidence for an impaired blood flow response to exercise engaging the leg muscles in

older postmenopausal women (Proctor *et al.* 2003; Parker *et al.* 2008). This altered vascular response may be attributable to the loss of sex hormones associated with menopause rather than age *per se* because exercise hyperaemia has been reported to be reduced in the early phase following the menopausal transition (Moore *et al.* 2012) and menopausal stage (and not age) appears to predict the vascular response to exercise in women (Parker *et al.* 2011). Based on these findings, we hypothesized that blood flow and O₂ delivery to the exercising leg would be lower in the postmenopausal women before training. However, we were not able to detect any differences in the haemodynamic response between the two groups. This finding of similar exercise hyperaemia is in contrast to observations made in the study by Moore *et al.* (2012) in which late peri- and early postmenopausal women displayed lower blood flow and vascular conductance than early perimenopausal women during knee-extensor exercise. This discrepancy may be explained by physiological changes associated with the transition from the pre- to the early perimenopausal phase because these changes may lead to compensatory vascular mechanisms and consequently enhanced exercise hyperaemia during the menopausal transition (Clarkson *et al.* 2013; Hodis *et al.* 2016). The finding that O₂ uptake and lactate release were similar in the two groups of women prior to training suggests that the matching of O₂ delivery and oxidative metabolism was not affected by the menopausal transition (Proctor & Moore, 2012). This preserved capacity of the circulation to deliver O₂ to meet the O₂ demand of the contracting muscles despite a marked reduction in vascular function, as assessed by arterial ACh and epoprostenol infusion, in the present group of postmenopausal women (Nyberg *et al.* 2016a) is probably the result of a greater contribution from redundant vasodilator mechanisms (Hellsten & Nyberg, 2015; Joyner & Casey, 2015). Because the function of the prostanoid system was found to be unaffected by the menopausal transition in the present group of women (Nyberg *et al.* 2016a), it is plausible that this system compensated for an impaired function of the NO system (Novella *et al.* 2012) given the reported redundancy between these vasodilator systems (Radegran & Saltin, 1999; Mortensen *et al.* 2007).

Experimental considerations

Despite strict inclusion criteria aiming to limit the confounding influence of age, it cannot be completely ruled out that the mean age difference of 4 years may have influenced study outcomes. However, the primary reason for the observed differences between the groups was probably the loss of oestrogens. We determined the content of relevant vasoactive enzymes and mitochondrial proteins in skeletal muscle samples in parallel with an assessment of the content of ER α , aiming to understand whether there

was a concomitant adaptation in the different proteins and whether the two groups of women responded differently. However, to what extent the observed changes in proteins had a direct functional consequence cannot be known because functional measures of vasoactive systems and mitochondrial respiration were not assessed. Lastly, the reduced sample size in the premenopausal group after training could also have impacted the study outcomes. However, the calculated power for this group was adequate and exclusion of the premenopausal women without post-training data did not change the outcome of the statistical analyses.

Conclusions

Aerobic high-intensity exercise training induced adaptations in leg vascular function and skeletal muscle mitochondrial proteins in pre- and recent postmenopausal women, with the greatest effect being attained in the postmenopausal women. This altered sensitivity to exercise training in postmenopausal women was accompanied by a greater upregulation of skeletal muscle ERR α and we propose that contraction-induced signalling through the ERR α pathway may be more pronounced in the absence of oestrogens. Furthermore, despite reductions in vascular function, the hyperaemic response to acute exercise is preserved in the early postmenopausal phase.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

MN, BS, JB and YH conceived and designed the experiments. MN, JE, CMM, CBA, KMBEH, IFG, NV, RFS and YH collected, analysed and interpreted the data. MN, JE, CMM, CBA, KMBEH, IFG, NV, RFS, BS, JB and YH drafted the article and revised it critically for important intellectual content. All authors have read and approved the final submission. All authors agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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