CALL FOR PAPERS | *Cardiovascular Mitochondria and Redox Control in Health and Disease*

Exercise training improves vascular mitochondrial function

Song-Young Park,[1,2] Matthew J. Rossman,[1,2] Jayson R. Gifford,[1,2] Leena P. Bharath,[2,5] Johann Bauersachs,[3] Russell S. Richardson,[1,2,4] E. Dale Abel,[5,6] J. David Symons,[2,5] and Christian Riehle[3,5,6]

[1]*Geriatric Research, Education, and Clinical Center, George E. Whalen Veterans Affairs Medical Center, Salt Lake City, Utah;* [2]*Department of Nutrition and Integrative Physiology, University of Utah, Salt Lake City, Utah;* [3]*Department of Cardiology and Angiology, Hannover Medical School, Hannover, Germany;* [4]*Division of Geriatrics, Department of Internal Medicine, University of Utah, Salt Lake City, Utah;* [5]*Division of Endocrinology, Metabolism and Diabetes, and Program in Molecular Medicine, University of Utah School of Medicine, Salt Lake City, Utah; and* [6]*Fraternal Order of Eagles Diabetes Research Center and Division of Endocrinology and Metabolism, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa*

Park SY, Rossman MJ, Gifford JR, Bharath LP, Bauersachs J, Richardson RS, Abel ED, Symons JD, Riehle C. Exercise training improves vascular mitochondrial function. *Am J Physiol Heart Circ Physiol* 310: H821–H829, 2016. First published January 29, 2016; doi:10.1152/ajpheart.00751.2015.—Exercise training is recognized to improve cardiac and skeletal muscle mitochondrial respiratory capacity; however, the impact of chronic exercise on vascular mitochondrial respiratory function is unknown. We hypothesized that exercise training concomitantly increases both vascular mitochondrial respiratory capacity and vascular function. Arteries from both sedentary (SED) and swim-trained (EX, 5 wk) mice were compared in terms of mitochondrial respiratory function, mitochondrial content, markers of mitochondrial biogenesis, redox balance, nitric oxide (NO) signaling, and vessel function. Mitochondrial complex I and complex I + II state 3 respiration and the respiratory control ratio (complex I + II state 3 respiration/complex I state 2 respiration) were greater in vessels from EX relative to SED mice, despite similar levels of arterial citrate synthase activity and mitochondrial DNA content. Furthermore, compared with the SED mice, arteries from EX mice displayed elevated transcript levels of *peroxisome proliferative activated receptor-γ coactivator-1α* and the downstream targets *cytochrome c oxidase subunit IV isoform 1*, *isocitrate dehydrogenase* (*Idh*) *2*, and *Idh3a*, increased manganese superoxide dismutase protein expression, increased endothelial NO synthase phosphorylation (Ser[1177]), and suppressed reactive oxygen species generation (all *P* < 0.05). Although there were no differences in EX and SED mice concerning endothelium-dependent and endothelium-independent vasorelaxation, phenylephrine-induced vasocontraction was blunted in vessels from EX compared with SED mice, and this effect was normalized by NOS inhibition. These training-induced increases in vascular mitochondrial respiratory capacity and evidence of improved redox balance, which may, at least in part, be attributable to elevated NO bioavailability, have the potential to protect against age- and disease-related challenges to arterial function.

mitochondria; vasculature; redox balance; arterial function

Address for reprint requests and other correspondence: C. Riehle, Dept. of Cardiology and Angiology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany (e-mail: riehle.christian@mh-hannover.de).

NEW & NOTEWORTHY

The impact of exercise training on vascular mitochondrial respiratory function is unknown. Here we show that arteries from swim-trained mice exhibit increased mitochondrial respiratory capacity, redox balance, and nitric oxide bioavailability compared with sedentary animals. These data confirm and extend the evidence that chronic physical activity is vasculoprotective.

WHILE MITOCHONDRIA COMPRISE ~10% of adult human body weight, likely due to differing metabolic demands, mitochondrial density is tissue specific. For example, the heart has a high metabolic demand, with mitochondria making up ~35% of cardiac tissue volume and being responsible for generating up to 90% of cardiac ATP requirements via β-oxidation (25, 37). In contrast, the vasculature has a relatively low metabolic demand, and mitochondria comprise only ~2–5% of the tissue volume, and ATP generation results largely from glycolysis (50). Our group recently documented that, when normalized for these large differences in mitochondrial content, mitochondrial respiratory capacity in cardiac, skeletal, and smooth muscle is very similar (37). Of particular importance to the current study, we also reported that complex I + II state 3 respiration was similar in intact and denuded vessels, suggesting that mitochondria located within the vascular smooth muscle, rather than the endothelium, are responsible for the majority of vascular respiration (37).

Evidence is accumulating that, rather than contributing to cellular respiration, an important role of the relatively small volume of mitochondria within the endothelium might be to act as signaling organelles that respond to cues from the local environment (50). Nitric oxide (NO), generated by endothelial nitric oxide synthase (eNOS), is a locally produced cue with documented potential to influence mitochondrial respiration in a number of cell types (e.g., brown adipocytes, 3T3-L1, U-937, and HeLa) (34, 35). Miller et al. (33) reported that, when basal NO generation was disrupted genetically (i.e., eNOS$^{+/-}$ and eNOS$^{-/-}$ mice) or pharmacologically [i.e., nitric oxide synthase (NOS) inhibition in rats via N^G-nitro-L-arginine methyl ester (L-NAME)], the protein abundance of the electron trans-

port chain (ETC) subunits was decreased in the aorta of these animals, relative to controls. These data support the potential role of basal NO generation in influencing mitochondrial capacity reported earlier in a variety of cell types (34, 35) and extend this important finding to include mitochondria located in the vasculature.

If mitochondrial respiration is suppressed by pharmacological and genetic approaches that limit basal NO production, but stimulated by NO donors (i.e., *S*-nitrosoacetyl penicillamine) (34), it is reasonable to hypothesize that a physiological intervention known to increase eNOS activity and NO generation (53), such as exercise training, might facilitate vascular mitochondrial respiration. Indeed, two investigations from the Reusch laboratory group (26, 33) reported that the content of one or more of the mitochondrial ETC subunits was greater in aortic lysates obtained from rats that performed treadmill running (8 days × 30–45 min/session) compared with their sedentary counterparts. Furthermore, the contribution from NOS was implicated by the finding that the adaptive mitochondrial response to exercise training was blunted in rats treated concurrently with L-NAME (33). While these insightful studies suggest that NO-mediated signaling is requisite for exercise to stimulate vascular mitochondrial adaptations, the impact of exercise training on mitochondrial respiratory capacity in the vasculature has not been directly assessed.

Consequently, the purpose of the present study was to test the hypothesis that exercise training concomitantly increases vascular mitochondrial respiratory capacity and vascular function. Arteries from both sedentary (SED) and exercise-trained (EX) male mice were compared in terms of mitochondrial respiratory function, vessel function, mitochondrial content, markers of mitochondrial biogenesis, redox balance, and NO signaling. Our results indicate that exercise training improves vascular mitochondrial respiratory capacity and redox balance, and evidence is provided that this adaptation is at least partly attributable to elevated NO bioavailability.

MATERIALS AND METHODS

Animals. Male C57BL/6J/129Sv mice were housed under controlled temperature (22°C) and light conditions (12:12-h light-dark cycle) and were provided with food and water ad libitum. All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Utah.

Exercise training and tissue collection. The 6-wk-old EX mice completed two swim-training sessions per day, each separated by at least a 4-h interval, for 36 days. The SED mice, of the same age, were also transported to the swim location two times per day, but remained in their cages. The duration of the initial session (10 min) was increased by 10 min each day until 90 min of continuous swimming was reached and maintained for an additional 28 days (24, 40). Mice were killed, and the entire aorta, both iliac arteries, and both femoral arteries were dissected free from adherent tissue while immersed in ice-cold dissection buffer that contained protease and phosphatase inhibitors (4, 45, 52). Vessel segments were used for the experiments described below.

Measurement of vascular mitochondrial respiration: aorta. Immediately upon excision the entire aorta was placed in ice-cold *buffer A* containing (in mM) 2.77 CaK$_2$EGTA, 7.23 K$_2$EGTA, 6.56 MgCl$_2$, 0.5 dithiothreitol (DTT), 50 K-MES, 20 imidazole, 20 taurine, 5.77 Na$_2$ATP, and 15 phosphocreatine at pH 7.1 for 30 min. Next, being shaken mildly for 40 min in *buffer A* supplemented with 50 μg/ml saponin, the aorta was rinsed (2 × 10 min/rinse) in *buffer B* containing (in mM) 2.77 CaK$_2$EGTA, 7.23 K$_2$EGTA, 6.56 MgCl$_2$, 0.5 DTT, 50

K-MES, 20 imidazole, 20 taurine, 5.77 ATP, and 15 phosphocreatine at pH 7.0 (37). Mitochondrial respiration was assessed by measuring the oxygen consumption rate in *buffer B*, while being continuously stirred, at 37°C using a Clark electrode (Hansatech, Kings Lynn, UK), as previously described (37). Briefly, after the baseline respiration rate in the absence of substrate was recorded: *1*) complex I state 2 respiration was assessed in the presence of glutamate + malate; *2*) complex I state 3 respiration, the ADP-stimulated state of oxidative phosphorylation, was measured in the presence of glutamate + malate + ADP; and *3*) complex I + II state 3 respiration was evaluated in the presence of glutamate + malate + ADP + succinate (37). In all experiments, the integrity of the outer mitochondrial membrane was confirmed by cytochrome *c* injection after the assessment of complex I and II state 3 respiration. None of the samples exhibited an increase in the rate of oxygen consumption following the addition of cytochrome *c* (data not shown). After respiration measurements, vessels were snap-frozen, and mitochondrial DNA content (mtDNA) and citrate synthase activity (CSA) were determined (29).

The respiratory control ratio (RCR) is defined as the ADP-stimulated flux divided by the flux without phosphorylation of ADP and was calculated as complex I + II state 3/complex I state 2 respiration. It should be noted that state 2 respiration was determined in the presence of glutamate + malate (in the absence of the complex II substrate succinate, since complex II does not release protons to the intermembrane space). Importantly, no difference was observed for state 2 respiration when comparing glutamate + malate + succinate vs. glutamate + malate as substrates (data not shown). For measurement of state 3 respiration, ADP together with succinate was supplemented to the respiration buffer to prevent depletion of metabolites from the mitochondrial matrix and to reconstitute the tricarboxylic acid cycle (14, 15, 37). Concentrations of each reagent in the vessel chamber were glutamate (2 mM), malate (10 mM), ADP (5 mM), succinate (10 mM), and cytochrome *c* (10 μM) (37).

Quantitative RT-polymerase chain reaction: Aorta. Total RNA was extracted from aortic tissue with TRIzol reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed (SuperScript III Reverse Transcriptase Kit; Invitrogen). Platinum Taq DNA polymerase (Invitrogen), primers, SYBR Green fluorescent dye (Invitrogen), and cDNA were transferred to a 384-well plate in triplicate, and real-time PCR was performed with an ABI Prism 7900HT instrument (Applied Biosystems, Foster City, CA) as previously described (39, 40). Data were normalized to ribosomal protein S16 (*Rps16*), and the results were expressed relative to the SED control mice. The following primers were used: *cytochrome c oxidase subunit IV isoform 1* (*Cox4i1*): forward 5′-CGCTGAAGGAGAAGGAGAAG-3′, reverse 5′-GCAGTGAAGCCAATGAAGAA-3′; *isocitrate dehydrogenase* (*Idh*) *2*: forward 5′-CCCTATTGCCAGCATCTTTG-3′, reverse 5′-TGTCCAGGAAGTCTGTGGTG-3′; *Idh3a*: forward 5′-CCCATC-CCAGTTTGATGTTC-3′, reverse 5′-GCATCATCACAGCACTA-AGCA-3′; *peroxisome proliferator activated receptor-γ coactivator-1α peroxisome proliferator activated receptor-γ coactivator-1α* (*Ppargc1a*) forward 5′-GTAAATCTGCGGGATGATGG-3′, reverse 5′-AGCAGGGTCAAAATCGTCTG-3′; and *Rps16*: forward 5′-TGCTGGTGTGGATATTCGGG-3′, reverse 5′-CCTTGAGAT-GGGCTTATCGG-3′.

Measurement of mtDNA content: Aorta. DNA was extracted from aortic segments using a phenol-chloroform-isoamyl alcohol solution (25:24:1) followed by ethanol precipitation. Next, DNA was dissolved in 100 μl of Tris-EDTA, and 5 μl of a 50 times dilution was subjected to real-time PCR amplification using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) in a total reaction volume of 25 μl containing 0.5 μM of each primer. Real-time PCR was performed with an iQ real-time PCR detection system (Bio-Rad, Hercules, CA). The mtDNA content relative to nuclear DNA content was determined using the following primers: mtDNA-encoded cytochrome *c* oxidase 1: forward 5′-ACTATACTACTACTAACAGACCG-3′, reverse 5′-GGTTCTTTTTTTTCCGGAGTA-3′; and nuclear DNA-encoded cy-

clophilin A: forward 5′-ACACGCCATAATGGCACTGG-3′, reverse 5′-CAGTCTTGGCAGTGCAGAT-3′ (5). Data were normalized relative to SED controls.

Measurement of CSA: Aorta and gastrocnemius. Frozen vessels previously used for mitochondrial respiration measurements were homogenized (in mM: 250 sucrose, 40 KCl, 2 EGTA, and 20 Tris·HCl, pH 7.4). The homogenates were then supplemented with 0.1% Triton X-100 and incubated on ice for 60 min followed by centrifugation for 8 min at 10,000 g and a 20 times dilution (37). Similarly, gastrocnemius muscle was homogenized followed by two freeze-thaw cycles to release the citrate synthase from the mitochondrial matrix, followed by centrifugation for 10 min and a 10× dilution (40). CSA was determined in a total reaction volume of 200 µl for vessel homogenates and 1 ml for skeletal muscle homogenates. The reaction was performed in reaction buffer containing (in mM) 220 sucrose, 40 KCl, 20 HEPES, 1 EGTA, 0.1 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), and 0.1 acetyl-CoA, pH 7.4 at 25°C, and was started by the addition of 0.05 mM oxaloacetate. CSA was monitored at 412 nm to detect the reaction of sulfhydryl groups of CoA with DTNB for a total duration of 3 min using an Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech).

Immunoblotting: Iliac and femoral arteries. Protein isolation and immunoblotting analyses were performed using both iliac arteries and segments of femoral arteries not used for vascular function experiments using procedures that have been previously described (4, 45, 52). With the exception of manganese superoxide dismutase (MnSOD; Enzo Life Sciences, Farmingdale, NY), all primary antibodies were obtained from Cell Signaling (Danvers, MA). Alexa Fluor anti-Mouse 680 or anti-Rabbit 680 (Invitrogen) were used as secondary antibodies, and fluorescence was quantified using the LICOR Odyssey imager (Lincoln, NE).

Measurement of reactive oxygen species: Iliac and femoral arteries. Reactive oxygen species (ROS) were quantified by 2′,7′-dichlorofluorescein diacetate (DCFDA) fluorescence. The assay is based on the conversion of nonfluorescent DCFDA to the highly fluorescent DCF in the presence of ROS. Tissue samples were homogenized in 0.05% Trypsin-EDTA (Invitrogen) and incubated for 30 min at 37°C. Following centrifugation (5 min at 14,000 g), pellets were incubated in DCFDA dissolved in DMEM (Invitrogen) at a final concentration of 5 µM for 30 min at 37°C. Samples were centrifuged for 5 min at 14,000 g, and pellets were resuspended in 200 µl of lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl$_2$, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 100 µM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) and incubated for 10 min at 4°C under constant agitation. Next, samples were centrifuged (5 min at 14,000 g), and supernatants were transferred to a black 96-well plate. DCF was determined by spectrophotometry using a fluorescence plate reader (Synergy HT Multi-Detection Reader; Bio-Tek Instruments, Winooski, VT) at 485 nm excitation/530 nm emission. Data were normalized to protein content, and results were expressed as a fold change relative to SED controls.

Measurement of vascular function: Femoral arteries. Vascular function was assessed in two femoral artery segments from each mouse using isometric tension techniques (4, 45, 52). First, both vessels completed a series of internal circumference-active tension curves to determine the vessel diameter that evoked the greatest tension development (L_{max}) to 100 mM KCl. Second, non-receptor-mediated and receptor-mediated vasocontractile responses to KCl (20–100 mM) and phenylephrine (PE, 10^{-8} to 10^{-5} M), respectively, were assessed. Third, after arteries were precontracted to ~65% of maximal PE-induced contraction and tension was stable (1, 4, 9, 45, 52), responses to acetylcholine (ACh, 10^{-8} to 10^{-6} M) were assessed to determine stimulated endothelium-dependent vasorelaxation. In a fourth protocol, one vessel from each mouse completed a second PE dose-response curve after a 10-min wash and a 20-min incubation with N^G-monomethyl-L-arginine (L-NMMA, 10^{-3} M). Results were

compared with the same vessel that was treated initially with PE in the absence of L-NMMA. Separate experiments revealed that repeatable contractions to PE are observed in femoral arteries when concentration-response curves are separated by 30 min. Specifically, maximal PE-evoked increases in tension development from baseline were not different when two dose-response curves were separated by 30 min (0.829 ± 0.058 vs. 0.911 ± 0.062 mg developed tension/µm vessel length). A fourth protocol completed on the second vessel obtained from each mouse involved administering 10^{-3} M L-NMMA to arteries that were stably precontracted with PE. L-NMMA-evoked vasocontraction in this context is a well-accepted functional estimate of basal eNOS activity (31) that we have used previously (45). The fifth and final protocol for both vessels involved administering sodium nitroprusside (10^{-9} to 10^{-4} M) to PE-precontracted arteries to determine endothelium-independent vasorelaxation. At least 30 min separated each protocol. When two vessel segments were treated identically, the results were averaged. All tension data were recorded continuously using an analog-to-digital interface card (Biopac Systems, Santa Barbara, CA) that allowed for subsequent off-line quantitative analyses.

Statistical analysis. Data are presented as means ± SE. Significance was accepted when $P < 0.05$. The comparison of one time point between two groups was made using an unpaired t-test. The comparison of multiple time points between groups was made using a one-way or two-way repeated-measures analysis of variance. Tukey's post hoc tests were performed when significant main effects were identified.

RESULTS

Efficacy of the exercise training regimen. CSA (nmol·min^{-1}·µg protein^{-1}) was greater in the gastrocnemius muscle obtained from the EX (44 ± 3) compared with the SED (32 ± 4) mice (Fig. 1*A*).

Vascular mitochondrial respiratory capacity in the EX and SED mice. Mitochondrial complex I, state 2 respiration was similar in the EX and SED mice (Fig. 1*B*). In contrast, mitochondrial complex I (11.9 ± 0.8 vs. 8.6 ± 1.2 pmol·mg^{-1}·s^{-1}) and complex I + II (20.8 ± 0.9 vs. 13.2 ± 1.3 pmol·mg^{-1}·s^{-1}; Fig. 1*B*), state 3 respiration, and RCR (2.5 ± 0.1 vs. 1.6 ± 0.1; Fig. 1*C*), were greater in vessels from EX compared with SED mice, respectively. Transcript levels of peroxisome proliferative activated receptor-γ coactivator-1α (PGC-1α; *Ppargc1a*) and the PGC-1α downstream targets involved in mitochondrial bioenergetics (*Cox4i1*, *Idh2*, *Idh3a*) were 1.3- to 1.5-fold greater in vessels from the EX compared with the SED mice (Fig. 1, *D* and *E*). Vascular mtDNA content (Fig. 1*F*) and CSA (Fig. 1*G*) were not different between groups.

Vascular redox balance in the EX and SED mice. MnSOD protein expression was approximately threefold greater (Fig. 2, *A* and *B*) and DCFDA fluorescence was 26% lower (Fig. 2*C*) in the vessels from the EX compared with the SED mice.

Vascular function and kinase signaling to eNOS in the EX and SED mice. There was no difference in vessel width (197 ± 6 and 221 ± 9 µm), vessel length (1,842 ± 23 and 1,846 ± 18 µm), resting tension at L_{max} (699 ± 10 and 713 ± 16 mg), or percent precontraction (68 ± 3 and 69 ± 4%), before the assessment of endothelium-dependent vasorelaxation, between the EX and SED mice, respectively. Neither endothelium-dependent (Fig. 3*A*) nor endothelium-independent (Fig. 3*B*) vasorelaxation was different when responses were compared among vessels from SED and EX mice. In contrast, non-receptor-mediated vasocontraction (Fig. 3*C*) tended to be greater in vessels from SED vs. EX mice at 80 mM ($P = 0.09$)
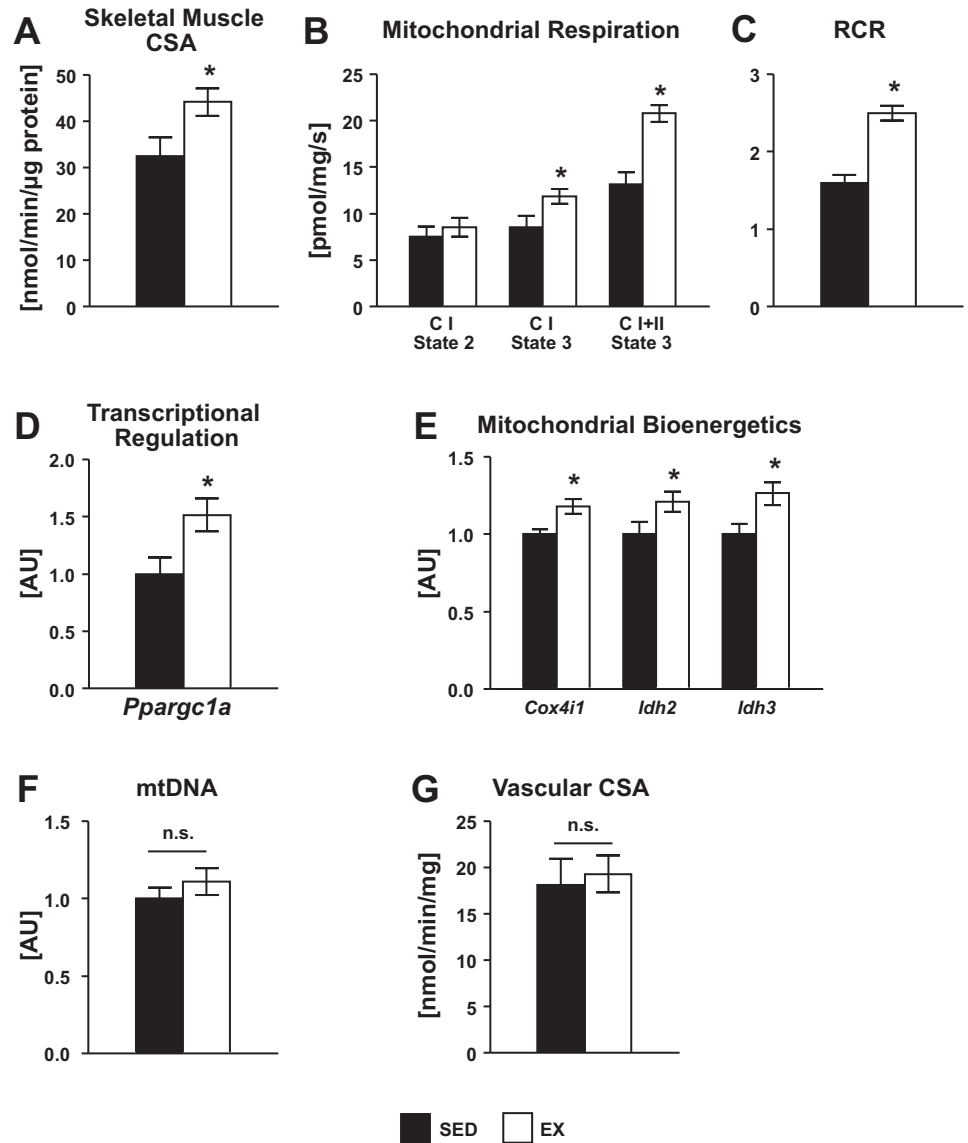
Fig. 1. Vascular mitochondrial respiratory capacity increases in response to exercise training. *A*: citrate synthase activity (CSA) in gastrocnemius muscle ($n = 6$). *B*: mitochondrial oxygen consumption in permeabilized aortas from exercise-trained (EX) and sedentary (SED) mice normalized to vessel wet weight. C I State 2, complex I state 2 respiration; C I State 3, complex I state 3 (following ADP stimulation); and C I + II State 3, complex I and II state 3 respiration (following ADP stimulation). *C*: respiratory control ratio (RCR), complex I + II state 3 normalized to complex I state 2 ($n = 8$ animals, 16 vessels). *D* and *E*: mRNA expression of *Ppargc1a* (*D*) and genes involved in mitochondrial bioenergetics (*E*) following EX ($n = 8$). Data are presented as a fold change relative to SED, $n = 8$. *F* and *G*: mitochondrial DNA (mtDNA) copy number (*F*) and CSA (*G*) in aortas ($n = 8$). $*P < 0.05$ vs. SED; ns, Not significant.

and 100 mM ($P = 0.06$) KCl (Fig. 3*C*), and receptor-mediated vasocontraction in response to PE was greater in arteries from the SED vs. EX mice ($P = 0.027$; Fig. 3*D*). PE-induced vasocontraction in both groups was greater ($P < 0.05$) in the presence vs. the absence of NOS inhibition using L-NMMA. However, no difference was observed between groups concerning PE-induced vasocontraction when results were compared in the presence of NOS inhibition ($P = 0.390$; Fig. 3*D*). Furthermore, L-NMMA caused a $16 \pm 4\%$ vs. $8 \pm 2\%$ increase ($P = 0.06$) in tension development in vessels from EX vs. SED mice, respectively.

In terms of kinase signaling to eNOS, arteries from the EX mice exhibited greater vascular phospho (p)-eNOS[Ser1177]/total eNOS, which was paralleled by greater phosphorylation of the upstream kinase p-Akt[Thr308]/total Akt and p-AMPK[Thr172]/total AMPK (Fig. 3, *E* and *F*).

DISCUSSION

The primary purpose of the present study was to determine the impact of exercise training on vascular mitochon-drial respiratory function. However, afforded by a combination of vessel function studies, the assessment of markers of mitochondrial biogenesis, redox balance, and NO signaling, we provide insight into the potential mechanisms and consequences of exercise training-induced mitochondrial and vascular adaptations. Specifically, despite evidence of unaltered mitochondrial content, vascular mitochondrial respiratory capacity was significantly greater in the EX mice, and this was accompanied by a greater RCR. In terms of vascular function, although the EX mice did not exhibit enhanced ACh-induced endothelial-mediated relaxation, evidence of greater NO bioavailability in arteries from EX vs. SED mice is provided. Finally, vessels from the EX mice exhibited greater MnSOD protein expression and suppressed ROS generation, which can be interpreted as evidence that exercise-induced oxidant stress was balanced by the appropriate antioxidant defense. These exercise training-induced increases in vascular mitochondrial respiratory capacity and evidence of improved redox balance, which were associated with elevated basal NO bioavailability, have the
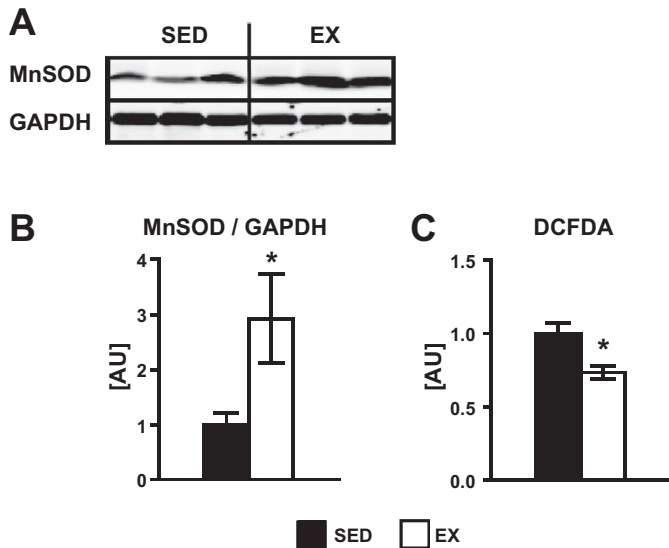
Fig. 2. Vascular indexes of antioxidant capacity following exercise training. *A* and *B*: manganese superoxide dismutase (MnSOD) protein expression normalized to GAPDH. *C*: reactive oxygen species (ROS) estimated by 2′,7′-dichlorofluorescein diacetate (DCFDA) fluorescence in iliac/femoral artery homogenates ($n = 8$). *$P < 0.05$ vs. SED.

potential to protect against age- and disease-related challenges to arterial function.

Vascular mitochondrial respiratory capacity and exercise training. Important roles for the mitochondria, e.g., oxygen sensing, calcium handling, and ROS production, are emerging and underscore the importance of mitochondrial quality control in health and disease. Prior studies have documented the exercise training-induced increase in mitochondrial respiratory capacity in both skeletal and cardiac muscle (23, 40). However, with only a few exceptions (19), the preponderance of data suggests that this exercise-induced increase in mitochondrial respiratory capacity is simply the consequence of an increase in mitochondrial content and not an inherent change in function of the mitochondrion (8, 16, 18, 21). Of note, the current CSA data from the gastrocnemius of the SED and EX mice, with a higher value in the EX mice, both support the concept of an exercise-induced increase in mitochondrial content in skeletal muscle and document the efficacy of the training intervention (Fig. 1*A*). The underlying mechanisms responsible for these exercise-induced mitochondrial adaptations in cardiac and skeletal muscle are incompletely understood, although it is becoming increasingly recognized that both PGC-1α and NO/cGMP-dependent pathways likely play a critical role (34, 35, 40). Similarly, aortic tissue from Sprague-Dawley rats (26) revealed a greater protein abundance of the ETC subunits as a consequence of 8 days of treadmill exercise. The PGC-1α-mediated transcriptional response to exercise training observed in the arteries of the EX mice in the current study provides a potential mechanism for the increased mitochondrial respiratory capacity following training (Fig. 1, *B–E*). However, in this study there was no evidence that the relatively mild increase in *Ppargc1a* resulted in mitochondrial biogenesis, as assessed by CSA and mtDNA content, but it is possible that this upregulation might have been sufficient to increase the mRNA expression of ETC subunits and mitochondrial respiratory capacity, independent of mitochondrial content. Importantly, the

current study is the first to document greater vascular mitochondrial respiratory capacity in response to exercise training using a functional ex vivo assay.

Vascular function, mitochondrial respiratory capacity, and exercise training. Originally we hypothesized that endothelium-dependent vasorelaxation would be greater in arteries from EX vs. SED mice. Instead, ACh-evoked vasorelaxation was similar between groups after the arteries were precontracted to the same extent using PE. While ACh-evoked vasorelaxation represents stimulated eNOS enzyme activity, it should be noted that several indexes of increased basal eNOS enzyme activity were observed in arteries from EX vs. SED mice. First, p-eNOS[Ser1177], a positive regulatory residue on the eNOS enzyme, was greater in arteries from EX vs. SED mice (Fig. 3, *E* and *F*). Second, exaggerated PE-induced vasocontraction observed in arteries from SED vs. EX animals was negated when dose-response curves were repeated in both groups in the presence of eNOS inhibition using L-NMMA (Fig. 3*D*). Third, L-NMMA-evoked vasocontraction of submaximally contracted arteries, a well-accepted functional estimate of basal eNOS activity (31), tended to be greater ($P = 0.06$) in vessels from EX vs. SED mice. Specifically, L-NMMA administration to PE-precontracted arteries evoked a $16 \pm 4\%$ vs. $8 \pm 2\%$ increase in tension development in vessels from EX vs. SED mice, respectively. In combination, these findings support the statement that endogenous opposition to vasocontraction afforded by basal NO was greater in arteries from EX vs. SED mice.

Interestingly, mitochondrial respiration is suppressed by pharmacological and genetic procedures that limit basal NO production, but is stimulated by NO donors in brown adipocytes, 3T3-L1, U-937, and HeLa cells. Furthermore, because 8 days of treadmill running increases mitochondrial ETC subunits in aortic lysates in a manner that is sensitive to NOS inhibition, we reasoned that a training-induced increase in basal NO generation might facilitate vascular mitochondrial respiration. Results from the present study are the first to reveal that training-induced increases in basal arterial NO production occur concurrently with elevations in vascular respiratory capacity. The mechanism(s) whereby vascular NO production might influence vascular respiratory capacity is complex and incompletely understood. For example, in addition to reports that basal NO production increases the expression of ETC subunits (26, 33), studies indicate that elevated NO concentrations inhibit cytochrome *c* oxidase activity to thereby impair mitochondrial respiratory capacity (6, 7). Also, it has been shown that mitochondrial depolarization activates NO synthase in endothelial cells to an extent that might link metabolic demand of exercise to vasodilation (22). The apparent paradoxical regulation of mitochondrial respiratory capacity by NO might be secondary to the intensity and/or nature of the stimulus. For example, mitochondrial respiration can be inhibited by high, but not low, concentrations of NO, and the effect of NO can be inhibited by light and thiols (6). Training studies that use different intensities and durations coupled with procedures to limit or exaggerate vascular NO production will be necessary to rigorously investigate this important issue.

Vascular mitochondrial function, redox balance, and exercise training. Mitochondrial dysfunction has been identified as a risk factor for vascular dysfunction and cardiovascular disease as a consequence of greater ROS production (28, 32, 47).
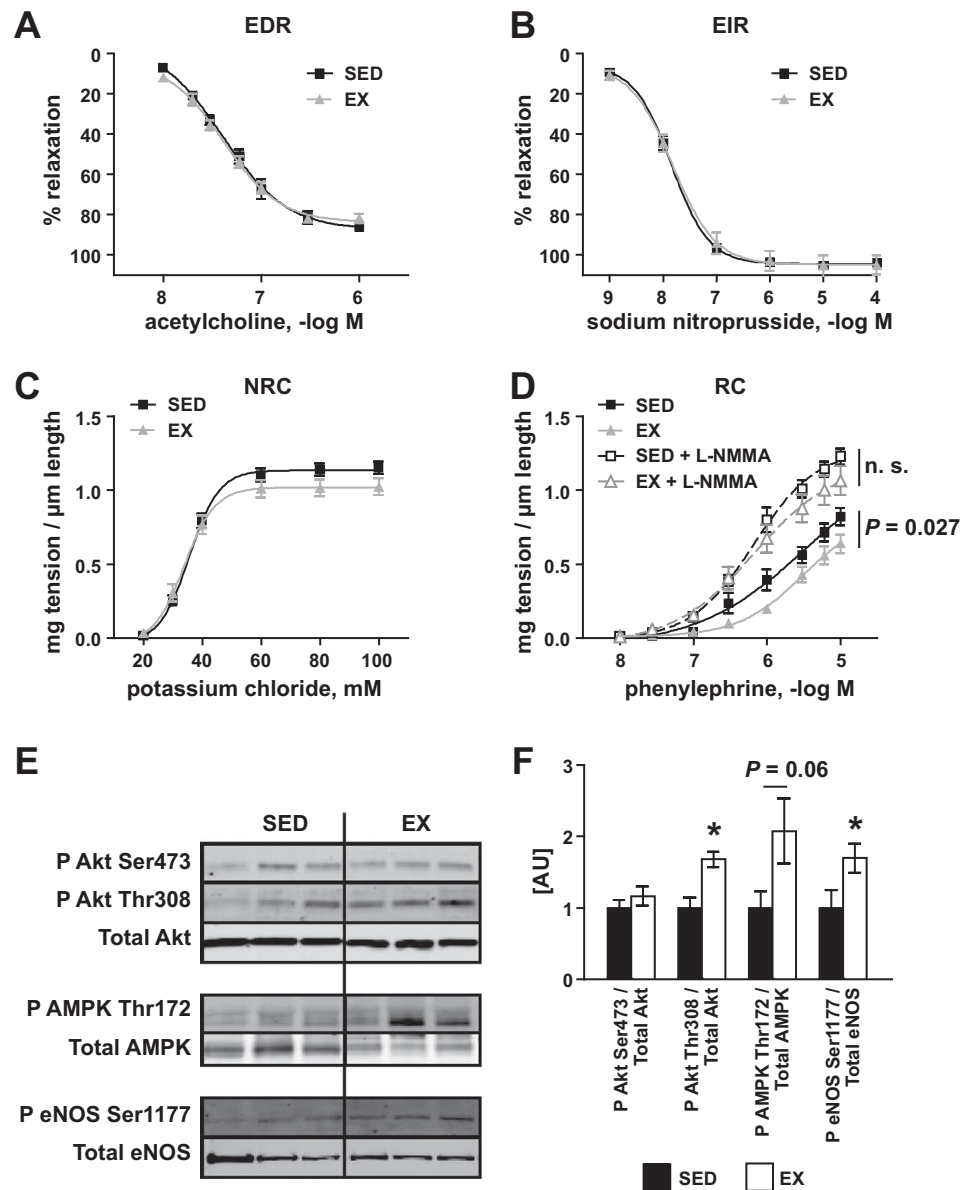
Fig. 3. Vascular function and kinase-mediated signaling to endothelial nitric oxide synthase (eNOS) following exercise training. *A* and *B*: *e*ndothelium-dependent (EDR, *A*) and independent (EIR, *B*) vasorelaxation. *C* and *D*: non-receptor-mediated (NRC, *C*) and receptor-mediated (RC, *D*) vasocontraction in the absence (*n* = 6–7 animals each, 19–23 vessels each) or presence of nitric oxide synthase inhibition *using* N^G-monomethyl-L-arginine (L-NMMA) (*n* = 7 animals, 9 vessels). *E* and *F*: representative Western blots from iliac/femoral artery homogenates (*E*) and densitometric quantification as indicated (*F*) (*n* = 7–8). Data are presented as fold change relative to SED: *$P < 0.05$ vs. SED.

Indeed, it has been suggested that attenuated mitochondrial content, in both vascular endothelial and smooth muscle cells, and the resultant increase in mitochondrial-derived ROS production may contribute to vascular dysfunction in aged rats (46). In the present study, we observed increased *Ppargc1a* and MnSOD expression and a decreased abundance of ROS following exercise training (Fig. 2, *A–C*), which, by either cause or effect, likely improve vascular redox balance and mitochondrial function. PGC-1α has been documented to regulate the expression of ROS-detoxifying enzymes, including MnSOD (43). Overexpression of PGC-1α in endothelial cells induced the expression of an antioxidant transcriptional program, resulting in increased mitochondrial detoxification capacity and decreased ROS levels. This was paralleled by increased mitochondrial membrane potential and decreased apoptotic cell death (42, 48). Furthermore, it has been determined that ROS formation plays a critical role for the positive effects of exercise training, including the induction of a PGC-1α-medi-

ated transcriptional program and an adaptive endogenous antioxidant response. This has been identified in human muscle samples as evidenced by an attenuated increase in PGC-1α and MnSOD expression following antioxidant supplementation with exercise training (41). While one study reported increased superoxide concentrations following exercise training (2), it is important to note that these data were obtained following a short-term exercise training protocol, and no chronic training effect was reported. The present study used a training regimen of 36 days total, which might account for the differences in the ROS levels observed when comparing these studies. In this context, the induction of the antioxidant response might compensate for the acute increase in ROS resulting in an overall improved redox balance. The present study uses two independent measurements indicating increased antioxidant defense i.e., increased expression of MnSOD and decreased cellular oxidative stress. While these assessments were obtained at the end of the study, it would be of great interest to investigate the

redox balance and antioxidant stress response at different time points throughout the training regimen, and this work is ongoing.

Acute exercise has been recognized to increase the phosphorylation and activation of protein kinase B (Akt), AMP-activated protein kinase (AMPK), and eNOS in the vasculature (53). Similar effects were observed in the present study following chronic exercise training, as also previously reported for cardiac tissue (51). In addition to the induction of an antioxidant response following acute training, increased ROS mediate the activation of Akt and AMPK signaling (2). We recently showed that AMPK is sufficient to activate p-eNOS^{Ser1177} in the presence of the phosphatidylinositol 3-kinase inhibitor wortmannin following acute training (51). Of note, in addition to Akt and AMPK, changes in blood flow activate eNOS, as observed in mesenteric arteries obtained from rats. These mechanisms are both Ca^{2+} dependent (eNOS translocation from the plasma membrane to intracellular compartments) and Ca^{2+} independent (via Akt-mediated eNOS phosphorylation) (10).

Experimental considerations. First, the current study was performed using healthy young mice, and it would be of great interest to ultimately investigate the impact of chronic exercise training on mitochondrial/vascular function and redox balance in the context of aging and/or disease. Second, our studies did not specifically address whether the mitochondrial and transcriptional changes we observed represent alterations within the vascular smooth muscle and/or endothelial cell compartment. Third, different segments of the vascular tree were used to investigate the endpoints reported. Because gene expression is differentially regulated throughout the vasculature (20, 36), we cannot exclude the possibility that exercise training might affect blood vessels in a heterogeneous manner, depending on their anatomical location. We acknowledge this limitation but highlight that comparisons between EX and SED mice in this study were performed in the same segment of the arterial tree. Finally, to definitively prove that NO increases vascular mitochondrial respiratory capacity, training studies are required using loss-of-function (e.g., eNOS null mice) and gain-of-function [e.g., eNOS transcription enhancers (12)] approaches. Ongoing studies in our laboratory are addressing each of these issues.

In conclusion, this study indicates that exercise training increases vascular mitochondrial respiratory capacity and improves vascular redox balance, and that these modifications correlate with elevated arterial NO bioavailability. These adaptations are relevant clinically because they have potential to protect against age- and disease-related challenges to arterial function, and our overall findings provide evidence that exercise training/physical activity is important to maintaining or improving vascular health.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.-Y.P., J.D.S., and C.R. conception and design of research; S.-Y.P., M.J.R., J.R.G., L.P.B., J.D.S., and C.R. performed experiments; S.-Y.P., L.P.B., J.D.S., and C.R. analyzed data; S.-Y.P., J.D.S., and C.R. interpreted results of experiments; S.-Y.P., J.D.S., and C.R. prepared figures; S.-Y.P., J.D.S., and C.R. drafted manuscript; S.-Y.P., J.B., R.S.R., E.D.A., J.D.S., and C.R. edited and revised manuscript; S.-Y.P., M.J.R., J.R.G., L.P.B., J.B., R.S.R., E.D.A., J.D.S., and C.R. approved final version of manuscript.

REFERENCES

1. **Alefishat E, Alexander SP, Ralevic V.** Antagonism of P2Y1-induced vasorelaxation by acyl CoA: a critical role for palmitate and 3′-phosphate. *Br J Pharmacol* 168: 1911–1922, 2013.
2. **Barbosa VA, Luciano TF, Marques SO, Vitto MF, Souza DR, Silva LA, Santos JP, Moreira JC, Dal-Pizzol F, Lira FS, Pinho RA, De Souza CT.** Acute exercise induce endothelial nitric oxide synthase phosphorylation via Akt and AMP-activated protein kinase in aorta of rats: role of reactive oxygen species. *Int J Cardiol* 167: 2983–2988, 2013.
3. **Bharath LP, Mueller R, Li Y, Ruan T, Kunz D, Goodrich R, Mills T, Deeter L, Sargsyan A, Anandh Babu PV, Graham TE, Symons JD.** Impairment of autophagy in endothelial cells prevents shear-stress-induced increases in nitric oxide bioavailability. *Can J Physiol Pharmacol* 92: 605–612, 2014.
4. **Bharath LP, Ruan T, Li Y, Ravindran A, Wan X, Nhan JK, Walker ML, Deeter L, Goodrich R, Johnson E, Munday D, Mueller R, Kunz D, Jones D, Reese V, Summers SA, Babu PV, Holland WL, Zhang QJ, Abel ED, Symons JD.** Ceramide-initiated protein phosphatase 2A activation contributes to arterial dysfunction in vivo. *Diabetes* 64: 3914–3926, 2015.
5. **Bonnard C, Durand A, Peyrol S, Chanseaume E, Chauvin MA, Morio B, Vidal H, Rieusset J.** Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest* 118: 789–800, 2008.
6. **Borutaite V, Budriunaite A, Brown GC.** Reversal of nitric oxide-, peroxynitrite- and S-nitrosothiol-induced inhibition of mitochondrial respiration or complex I activity by light and thiols. *Biochim Biophys Acta* 1459: 405–412, 2000.
7. **Brown GC.** Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim Biophys Acta* 1504: 46–57, 2001.
8. **Chilibeck PD, Syrotuik DG, Bell GJ.** The effect of concurrent endurance and strength training on quantitative estimates of subsarcolemmal and intermyofibrillar mitochondria. *Int J Sports Med* 23: 33–39, 2002.
9. **Dooley LM, Abdalmula A, Washington EA, Kaufman C, Tudor EM, Ghosh P, Itescu S, Kimpton WG, Bailey SR.** Effect of mesenchymal precursor cells on the systemic inflammatory response and endothelial dysfunction in an ovine model of collagen-induced arthritis. *PLos One* 10: e0124144, 2015.
10. **Figueroa XF, Gonzalez DR, Puebla M, Acevedo JP, Rojas-Libano D, Duran WN, Boric MP.** Coordinated endothelial nitric oxide synthase activation by translocation and phosphorylation determines flow-induced nitric oxide production in resistance vessels. *J Vasc Res* 50: 498–511, 2013.

11. **Fleming I, Bauersachs J, Schafer A, Scholz D, Aldershvile J, Busse R.** Isometric contraction induces the Ca2+-independent activation of the endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 96: 1123–1128, 1999.

12. **Fraccarollo D, Widder JD, Galuppo P, Thum T, Tsikas D, Hoffmann M, Ruetten H, Ertl G, Bauersachs J.** Improvement in left ventricular remodeling by the endothelial nitric oxide synthase enhancer AVE9488 after experimental myocardial infarction. *Circulation* 118: 818–827, 2008.

13. **Gielen S, Schuler G, Adams V.** Cardiovascular effects of exercise training: molecular mechanisms. *Circulation* 122: 1221–1238, 2010.

14. **Gifford JR, Trinity JD, Layec G, Garten RS, Park SY, Rossman MJ, Larsen S, Dela F, Richardson RS.** Quadriceps exercise intolerance in patients with chronic obstructive pulmonary disease: the potential role of altered skeletal muscle mitochondrial respiration. *J Appl Physiol* 119: 882–888, 2015.

15. **Gnaiger E.** Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. *In J Biochem Cell Biol* 41: 1837–1845, 2009.

16. **Gram M, Dahl R, Dela F.** Physical inactivity and muscle oxidative capacity in humans. *Eur J Sport Sci* 14: 376–383, 2014.

17. **Green DJ, Swart A, Exterkate A, Naylor LH, Black MA, Cable NT, Thijssen DH.** Impact of age, sex and exercise on brachial and popliteal artery remodelling in humans. *Atherosclerosis* 210: 525–530, 2010.

18. **Hoppeler H, Fluck M.** Plasticity of skeletal muscle mitochondria: structure and function. *Med Sci Sports Exercise* 35: 95–104, 2003.

19. **Jacobs RA, Lundby C.** Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes. *J Appl Physiol* 114: 344–350, 2013.

20. **Jenkins NT, Padilla J, Thorne PK, Martin JS, Rector RS, Davis JW, Laughlin MH.** Transcriptome-wide RNA sequencing analysis of rat skeletal muscle feed arteries. I. Impact of obesity. *J Appl Physiol* 116: 1017–1032, 2014.

21. **Jubrias SA, Esselman PC, Price LB, Cress ME, Conley KE.** Large energetic adaptations of elderly muscle to resistance and endurance training. *J Appl Physiol* 90: 1663–1670, 2001.

22. **Katakam PV, Wappler EA, Katz PS, Rutkai I, Institoris A, Domoki F, Gaspar T, Grovenburg SM, Snipes JA, Busija DW.** Depolarization of mitochondria in endothelial cells promotes cerebral artery vasodilation by activation of nitric oxide synthase. *Arterioscler Thromb Vasc Biol* 33: 752–759, 2013.

23. **Kim B, Lee H, Kawata K, Park JY.** Exercise-mediated wall shear stress increases mitochondrial biogenesis in vascular endothelium. *PLos One* 9: e111409, 2014.

24. **Kim J, Wende AR, Sena S, Theobald HA, Soto J, Sloan C, Wayment BE, Litwin SE, Holzenberger M, LeRoith D, Abel ED.** Insulin-like growth factor I receptor signaling is required for exercise-induced cardiac hypertrophy. *Mol Endocrinol* 22: 2531–2543, 2008.

25. **Knaapen P, Germans T, Knuuti J, Paulus WJ, Dijkmans PA, Allaart CP, Lammertsma AA, Visser FC.** Myocardial energetics and efficiency: current status of the noninvasive approach. *Circulation* 115: 918–927, 2007.

26. **Knaub LA, McCune S, Chicco AJ, Miller M, Moore RL, Birdsey N, Lloyd MI, Villarreal J, Keller AC, Watson PA, Reusch JE.** Impaired response to exercise intervention in the vasculature in metabolic syndrome. *Diabetes Vasc Dis* 10: 222–238, 2013.

27. **Larsen RG, Callahan DM, Foulis SA, Kent-Braun JA.** Age-related changes in oxidative capacity differ between locomotory muscles and are associated with physical activity behavior. *Appl Physiol Nutr Metab* 37: 88–99, 2012.

28. **Larsen S, Hey-Mogensen M, Rabol R, Stride N, Helge JW, Dela F.** The influence of age and aerobic fitness: effects on mitochondrial respiration in skeletal muscle. *Acta Physiol (Oxf)* 205: 423–432, 2012.

29. **Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, Schroder HD, Boushel R, Helge JW, Dela F, Hey-Mogensen M.** Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol* 590: 3349–3360, 2012.

30. **Laughlin MH, Newcomer SC, Bender SB.** Importance of hemodynamic forces as signals for exercise-induced changes in endothelial cell phenotype. *J Appl Physiol* 104: 588–600, 2008.

31. **Lefer AM, Ma XL.** Decreased basal nitric oxide release in hypercholesterolemia increases neutrophil adherence to rabbit coronary artery endothelium. *Arterioscler Thromb* 13: 771–776, 1993.

32. **Madamanchi NR, Runge MS.** Mitochondrial dysfunction in atherosclerosis. *Circ Res* 100: 460–473, 2007.

33. **Miller MW, Knaub LA, Olivera-Fragoso LF, Keller AC, Balasubramaniam V, Watson PA, Reusch JE.** Nitric oxide regulates vascular adaptive mitochondrial dynamics. *Am J Physiol Heart Circ Physiol* 304: H1624–H1633, 2013.

34. **Nisoli E, Carruba MO.** Nitric oxide and mitochondrial biogenesis. *J Cell Sci* 119: 2855–2862, 2006.

35. **Nisoli E, Falcone S, Tonello C, Cozzi V, Palomba L, Fiorani M, Pisconti A, Brunelli S, Cardile A, Francolini M, Cantoni O, Carruba MO, Moncada S, Clementi E.** Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. *Proc Natl Acad Sci USA* 101: 16507–16512, 2004.

36. **Padilla J, Jenkins NT, Thorne PK, Martin JS, Rector RS, Davis JW, Laughlin MH.** Transcriptome-wide RNA sequencing analysis of rat skeletal muscle feed arteries. II. Impact of exercise training in obesity. *J Appl Physiol* 116: 1033–1047, 2014.

37. **Park SY, Gifford JR, Andtbacka RH, Trinity JD, Hyngstrom JR, Garten RS, Diakos NA, Ives SJ, Dela F, Larsen S, Drakos S, Richardson RS.** Cardiac, skeletal, and smooth muscle mitochondrial respiration: are all mitochondria created equal? *Am J Physiol Heart Circ Physiol* 307: H346–H352, 2014.

38. **Picard M, Csukly K, Robillard ME, Godin R, Ascah A, Bourcier-Lucas C, Burelle Y.** Resistance to Ca2+-induced opening of the permeability transition pore differs in mitochondria from glycolytic and oxidative muscles. *Am J Physiol Regul Integr Comp Physiol* 295: R659–R668, 2008.

39. **Riehle C, Wende AR, Sena S, Pires KM, Pereira RO, Zhu Y, Bugger H, Frank D, Bevins J, Chen D, Perry CN, Dong XC, Valdez S, Rech M, Sheng X, Weimer BC, Gottlieb RA, White MF, Abel ED.** Insulin receptor substrate signaling suppresses neonatal autophagy in the heart. *J Clin Invest* 123: 5319–5333, 2013.

40. **Riehle C, Wende AR, Zhu Y, Oliveira KJ, Pereira RO, Jaishy BP, Bevins J, Valdez S, Noh J, Kim BJ, Moreira AB, Weatherford ET, Manivel R, Rawlings TA, Rech M, White MF, Abel ED.** Insulin receptor substrates are essential for the bioenergetic and hypertrophic response of the heart to exercise training. *Mol Cell Biol* 34: 3450–3460, 2014.

41. **Ristow M, Zarse K, Oberbach A, Kloting N, Birringer M, Kiehntopf M, Stumvoll M, Kahn CR, Bluher M.** Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci USA* 106: 8665–8670, 2009.

42. **Rowe GC, Jiang A, Arany Z.** PGC-1 coactivators in cardiac development and disease. *Circ Res* 107: 825–838, 2010.

43. **St.-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, Spiegelman BM.** Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 127: 397–408, 2006.

44. **Symons JD, Hayashi Y, Ensunsa JL.** Improved coronary vascular function evoked by high-intensity treadmill training is maintained in arteries exposed to ischemia and reperfusion. *J Appl Physiol* 95: 1638–1647, 2003.

45. **Symons JD, McMillin SL, Riehle C, Tanner J, Palionyte M, Hillas E, Jones D, Cooksey RC, Birnbaum MJ, McClain DA, Zhang QJ, Gale D, Wilson LJ, Abel ED.** Contribution of insulin and Akt1 signaling to endothelial nitric oxide synthase in the regulation of endothelial function and blood pressure. *Circ Res* 104: 1085–1094, 2009.

46. **Ungvari Z, Labinskyy N, Gupte S, Chander PN, Edwards JG, Csiszar A.** Dysregulation of mitochondrial biogenesis in vascular endothelial and smooth muscle cells of aged rats. *Am J Physiol Heart Circ Physiol* 294: H2121–H2128, 2008.

47. **Ungvari Z, Sonntag WE, Csiszar A.** Mitochondria and aging in the vascular system. *J Mol Med* 88: 1021–1027, 2010.

48. **Valle I, Alvarez-Barrientos A, Arza E, Lamas S, Monsalve M.** PGC-1alpha regulates the mitochondrial antioxidant defense system in vascular endothelial cells. *Cardiovasc Res* 66: 562–573, 2005.

49. **Vettor R, Valerio A, Ragni M, Trevellin E, Granzotto M, Olivieri M, Tedesco L, Ruocco C, Fossati A, Fabris R, Serra R, Carruba MO, Nisoli E.** Exercise training boosts eNOS-dependent mitochondrial biogenesis in mouse heart: role in adaptation of glucose metabolism. *Am J Physiol Endocrinol Metab* 306: E519–E528, 2014.

50. **Webb RC.** Smooth muscle contraction and relaxation. *Adv Physiol Educ* 27: 201–206, 2003.

51. **Yang L, Jia Z, Yang L, Zhu M, Zhang J, Liu J, Wu P, Tian W, Li J, Qi Z, Tang X.** Exercise protects against chronic beta-adrenergic remod-

eling of the heart by activation of endothelial nitric oxide synthase. *PLos One* 9: e96892, 2014.

52. **Zhang QJ, Holland WL, Wilson L, Tanner JM, Kearns D, Cahoon JM, Pettey D, Losee J, Duncan B, Gale D, Kowalski CA, Deeter N, Nichols A, Deesing M, Arrant C, Ruan T, Boehme C, McCamey DR, Rou J, Ambal K, Narra KK, Summers SA, Abel ED, Symons JD.** Ceramide mediates vascular dysfunction in diet-induced obesity by PP2A-mediated dephosphorylation of the eNOS-Akt complex. *Diabetes* 61: 1848–1859, 2012.

53. **Zhang QJ, McMillin SL, Tanner JM, Palionyte M, Abel ED, Symons JD.** Endothelial nitric oxide synthase phosphorylation in treadmill-running mice: role of vascular signalling kinases. *J Physiol* 587: 3911–3920, 2009.