Effects of ageing and exercise training on eNOS uncoupling in skeletal muscle resistance arterioles

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Reduced availability of tetrahydrobiopterin (BH_4) contributes to the age-related decline of nitric oxide (NO)-mediated vasodilatation of soleus muscle arterioles. Depending on availability of substrate and/or necessary co-factors, endothelial nitric oxide synthase (eNOS) can generate NO and/or superoxide (O_2^-). We evaluated the effects of age and chronic exercise on flow-induced vasodilatation and levels of NO and O_2^- in soleus muscle arterioles. Young (3 months) and old (22 months) male rats were exercise trained or remained sedentary (SED) for 10 weeks. Flow-stimulated NO and O_2^-, as well as BH_4 and L-arginine content, were determined in soleus muscle arterioles. Flow-induced vasodilatation was assessed under control conditions and during the blockade of O_2^- and/or hydrogen peroxide. Exercise training enhanced flow-induced vasodilatation in arterioles from young and old rats. Old age reduced, and exercise training restored, BH_4 content and flow-stimulated NO availability. Flow-stimulated, eNOS-derived O_2^- levels were higher in arterioles from old SED compared to those from young SED rats. Exercise training increased flow-stimulated eNOS-derived O_2^- levels in arterioles from young but not old rats. O_2^- scavenging with Tempol reduced flow-induced vasodilatation from all groups except young SED rats. Addition of catalase to Tempol-treated arterioles eliminated flow-induced vasodilatation in arterioles from all groups. Catalase reduced flow-induced vasodilatation from all groups. In Tempol-treated arterioles, flow-induced vasodilatation was restored by deferoxamine, an iron chelator. These data indicate that uncoupling of eNOS contributes to the age-related decline in flow-induced vasodilatation; however, reactive oxygen species are required for flow-induced vasodilatation in soleus muscle arterioles from young and old rats.

Abbreviations DHE, dihydroethidium; eNOS, endothelial nitric oxide synthase; ET, exercise trained; NO, nitric oxide; ROS, reactive oxygen species; SED, sedentary; SOD, superoxide dismutase.

Maximal exercise capacity declines with advancing age (Ogawa *et al.* 1992; Fitzgerald *et al.* 1997) and although part of this decline is associated with a reduction of maximal cardiac output (Lakatta, 1995), alterations in the local control of skeletal muscle blood flow and declines in vascular conductance also contribute to this abatement (Dinenno *et al.* 1999). Numerous studies have demonstrated that age impairs endothelial function in conduit and skeletal muscle resistance arteries in human and animal models (Gerhard *et al.* 1996; DeSouza *et al.* 2000; Muller-Delp *et al.* 2002; Woodman *et al.* 2002).

Old age-associated reductions in endothelium-dependent dilatation of skeletal muscle resistance arterioles occur in part through reduced nitric oxide (NO) signalling (Spier *et al.* 2004). Mechanisms that may underlie this age-associated impairment in NO signalling include reduced availability of substrate (L-arginine) (Morris, 2000) or cofactors (e.g. tetrahydrobiopterin (BH_4)) (Cosentino & Katusic, 1995; Tiefenbacher, 2001), reduced endothelial NO synthase (eNOS) protein levels and/or activity, and increased superoxide production (Darley-Usmar *et al.* 1995). Depending on the availability

of L-arginine and/or BH_4, eNOS can become uncoupled, resulting in the generation of NO and superoxide (O_2^-) (Vasquez-Vivar *et al.* 1998; Cosentino & Luscher, 1999). Ageing-induced diminution of BH_4 contributes to the decline of NO-mediated dilatation to flow, and sepiapterin, a precursor to BH_4 synthesis (Bagi *et al.* 2004), improves flow-induced vasodilatation in soleus muscle arterioles of aged rats (Delp *et al.* 2008). BH_4 supplementation ameliorates endothelium-dependent dilatation in humans with cardiovascular diseases or elevated risk factors (Stroes *et al.* 1997; Maier *et al.* 2000); however, the impact of reduced BH_4 availability on eNOS function has not been directly evaluated in the skeletal muscle resistance vasculature.

Reactive oxygen species (ROS) potentially decrease NO bioavailability by two mechanisms. First, O_2^- rapidly interacts with NO to form peroxynitrite ($ONOO^-$), a potent ROS, which elicits cellular damage (Darley-Usmar *et al.* 1995; Cai & Harrison, 2000). Secondly, the presence of ROS, and primarily $ONOO^-$, reduces BH_4 to the inactive trihydrobiopterin radical (BH_3), thereby reducing this necessary co-factor and subsequent NO production. Recent reports indicate that insufficient BH_4 is the major determinant of whether eNOS produces O_2^- or NO (Bevers *et al.* 2006; Crabtree *et al.* 2009).

Aerobic exercise training restores age-associated reductions in NO-mediated dilatation in human and animal models (Taddei *et al.* 1995; DeSouza *et al.* 2000; Spier *et al.* 2004). Exercise training may increase NO bioavailability, in part, through enhanced regulation of ROS (Rush *et al.* 2000) or through increased expression of eNOS protein (Spier *et al.* 2004); however, the effects of age and exercise training on the regulation of eNOS activity have not been evaluated. Eskurza *et al.* (2005) demonstrated that an acute bolus of BH_4 augments endothelial function in the brachial artery of old sedentary men, but had no effect on endothelium-dependent dilatation in habitually active, age-matched counterparts. These data suggest that aerobic exercise training prevents the loss of NO bioavailability by preserving BH_4 in aged individuals; however, the effects of exercise training on BH_4 levels in the resistance vasculature of skeletal muscle have not been determined. Here, we investigate the effects of age and exercise training on BH_4 availability and putative eNOS uncoupling in soleus muscle arterioles of male rats.

Therefore, the purpose of this study was threefold. First, we sought to determine whether age-induced reductions of arteriolar BH_4 are associated with eNOS uncoupling. Second, we investigated the possibility that exercise training restores arteriolar BH_4 availability and reverses eNOS uncoupling with old age. And third, we determined whether eNOS uncoupling in skeletal muscle arterioles is accompanied by significant increases in ROS and a concomitant decrease in NO bioavailability.

Methods

Animals

All procedures in this study were approved by the Institutional Animal Care and Use Committees at West Virginia University. All methods complied fully with guidelines set in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, revised 1996). Young (3 months) and old (22 months) male Fischer 344 rats were obtained from Harlan (Indianapolis, IN, USA), housed under a 12 : 12 h light–dark cycle, and given food and water *ad libitum*. This particular strain was chosen because cardiovascular function decreases with age in these rats, without the development of atherosclerosis or hypertension (Lakatta, 1995).

Exercise training

All rats were habituated to treadmill exercise, during which each rat walked on a motor-driven treadmill at 5 m min^{-1} (0 deg incline), 5 min per day for 3 days. After habituation, young and old rats were randomly assigned to either a control sedentary (SED) group (young SED, $n = 30$, and old SED, $n = 32$) or an exercise-trained (ET) group (young ET, $n = 37$, and old ET, $n = 26$). ET rats performed treadmill running at 15 m min^{-1} (15 deg incline), 5 days per week, for 10–12 weeks. The duration of running was gradually increased in the first 3 weeks until a 60 min duration was reached. The rats continued to run 5 days per week for 60 min per day for the remainder of the 10–12 week training period. Vascular responses were determined at least 48 h after the last exercise bout in ET rats.

Muscle oxidative enzyme activity

To determine the efficacy of the training protocol, sections of soleus muscle were stored at $-80°C$ for determination of citrate synthase activity, a measure of muscle oxidative capacity (Srere, 1969; Delp & Duan, 1996).

Microvessel preparation

Rats were anaesthetized with isoflurane (5%, O_2 balance) and killed by decapitation. The gastrocnemius–plantaris–soleus muscle group was dissected free from both hindlimbs and placed in a cold (4°C), filtered physiological saline solution (PSS) containing 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM Mops buffer, and 1 g/100 ml BSA, pH 7.4. With the aid of a dissecting microscope (Olympus SVH10), first-order (1A) arterioles were isolated and dissected from the soleus muscle, which is composed

primarily of high-oxidative fibres (Delp & Duan, 1996), as previously described (Muller-Delp *et al.* 2002; Spier *et al.* 2004). The arterioles were then transferred to a Lucite chamber containing PSS with 1% albumin (pH 7.4) equilibrated with room air. Each end of the arteriole was cannulated with micropipettes filled with PSS-albumin solution and secured with a nylon suture. The sizes and resistances of the pipettes were matched to within 1%. The chamber was placed on the stage of an inverted microscope (Zeiss Axio40), equipped with a video camera (Panasonic BP310), video caliper (Colorado Video), and data-acquisition system (PowerLab; ADInstruments). Arterioles were pressurized via two independent reservoirs and checked for leaks. If leaks were present, the arterioles were discarded. Vessels that were free from leaks were pressurized to 70 cmH$_2$O, gradually warmed to 37°C, and allowed to develop spontaneous tone during an initial equilibration period. The bathing solution was changed every 20 min during the course of the experiment.

Vasodilator responses to intraluminal flow

Upon displaying a steady level of spontaneous tone, arterioles were exposed to graded increases in intraluminal flow in the absence of changes in intraluminal pressure (Muller-Delp *et al.* 2002; Delp *et al.* 2008). This was accomplished by altering the heights of independent pressure reservoirs in equal and opposite directions so that a pressure difference was created across the vessel without altering mean intraluminal pressure. Diameter measurements were determined in response to incremental pressure differences of 2, 4, 10, 20, 40 and 60 cmH$_2$O. Volumetric flow (Q) was then calculated from inner diameter (d) and mean red cell velocity (V_{rbc}), according the following equation (Davis, 1987; Kuo *et al.* 1990; Muller-Delp *et al.* 2002):

$$Q = \pi (V_{rbc}/1.6)(d/2)^2$$

At the end of the experiment, arterioles were placed in Ca^{2+}-free PSS with 100 μM of the NO donor, sodium nitroprusside for 1 h to obtain the maximal passive diameter (Muller-Delp *et al.* 2002; Spier *et al.* 2004).

Effects of ROS scavengers on flow-induced vasodilatation

To determine the role of O$_2^-$, hydrogen peroxide (H$_2$O$_2$), and hydroxyl ion (OH$^-$) in old age-related reductions of flow-induced vasodilatation, responses to flow were evaluated in the presence of the following: (1) a superoxide dismutase mimetic, Tempol (100 μM; Sigma) (Chen *et al.* 2007), (2) a H$_2$O$_2$ scavenger, catalase (100 U; Sigma) (Emsley *et al.* 1999), (3) Tempol (100 μM) plus catalase (100 U), (4) a NADPH oxidase inhibitor, apocynin

(100 μM; Sigma) (Zhu *et al.* 2007), and (5) Tempol plus deferoxamine (100 μM; Sigma) (Thengchaisri *et al.* 2006), an iron chelator, and inhibitor of OH$^-$ formation (Thengchaisri *et al.* 2006).

Detection of flow-stimulated NO availability

To evaluate flow-stimulated bioavailability of NO, isolated soleus muscle arterioles were cannulated as described above. 4,5-Diaminofluorescein diacetate (DAF-2DA) (Calbiochem) is a non-fluorescent dye until it reacts with NO in the presence of oxygen to form a fluorescent compound, triazolofluorescein (2DAF-2T) (Kojima *et al.* 1998). The intensity of the fluorescence signal is proportional to NO levels. Following intraluminal loading of DAF-2DA (2.5 μM) for 20 min, soleus muscle arterioles were exposed to a flow rate of 34 nl s^{-1} and fluorescence images were acquired every 15 s over 2 min. To confirm that availability of NO occurred through eNOS, flow-induced DAF-2DA fluorescence was evaluated in the presence of N^G-nitro-L-arginine methyl ester (L-NAME). DAF-2DA fluorescence during exposure to 10 μM Dea-NONOate, an NO donor, was used as a positive control to ensure that the dye was not saturated and to determine maximal fluorescence intensity as a control for loading of dye. A user-defined region of interest (ROI), which encompassed the entire vessel diameter and the central portion of the length of each vessel, was analysed for fluorescence intensity using ImageJ software (National Institutes of Health). Basal fluorescence levels varied among ROIs and in different experiments. Therefore, after background subtraction, changes in fluorescence intensity were expressed as the ratio of DAF stimulated/DAF at baseline. Both baseline and flow-induced fluorescence intensity values were measured within the same ROI in the same focal plane of each vessel.

Detection of flow-stimulated O$_2^-$ generation

Dihydroethidium (DHE) (Calbiochem) fluorescence was used to evaluate flow-stimulated O$_2^-$ generation in real time (Suzuki *et al.* 1995). To evaluate O$_2^-$ generation in response to flow stimulation, soleus muscle arterioles were cannulated as described above. DHE fluorescence was evaluated in arterioles similar to those described by Suzuki *et al.* (1995). Following intraluminal loading of DHE (40 μM) for 20 min, soleus muscle arterioles were exposed to a flow rate of 34 nl s^{-1} and fluorescence images were acquired every 15 s over 2 min. The specificity of the DHE dye for superoxide was confirmed by application of the superoxide dismutase (SOD) mimetic, Tempol, which significantly reduced DHE fluorescence. To determine whether generation of O$_2^-$ occurred through eNOS, flow-stimulated DHE fluorescence was evaluated in the

presence of L-NAME. DHE fluorescence during exposure to 100% ethanol was used as a positive control to ensure that the dye was not saturated and to control for equal loading of dye. A region of interest was selected for each vessel and changes in fluorescence intensity for each respective ROI were analysed using ImageJ software as described above.

Arteriolar L-arginine and BH$_4$ measurements

Arteriolar L-arginine and BH$_4$ levels were determined using the HPLC method as previously described (Wu & Meininger, 1995; Meininger *et al.* 2000; Delp *et al.* 2008). Briefly, soleus muscle arterioles were dissected from each animal and pooled. For L-arginine analysis, vessels (10 to 15 mg) were homogenized with 0.2 ml 1.5 M HClO$_4$, and then 0.1 ml 2 M K$_2$CO$_3$ was added. The homogenates were centrifuged at 10 000 *g* for 1 min, and an aliquot (0.2 ml) of the supernatant was used for sample determination of L-arginine content (Wu & Meininger, 1995). For BH$_4$ analysis, arterioles (10 to 15 mg) were homogenized in 0.1 ml 0.1 M phosphoric acid containing 5 mM dithioerythritol (an antioxidant), to which 17.5 μl 2 M trichloroacetic acid was added. Extracts were oxidized with acidic or basic iodine. Acidic oxidation quantitatively converts BH$_4$ and dihydrobiopterin to biopterin; basic oxidation converts dihydrobiopterin and BH$_4$ to biopterin and pterin, respectively. Samples were incubated in the dark for 1 h. Excess iodine was removed by adding ascorbic acid (final concentration, 0.1 M). The final solution was analysed on a C18 reversed-phase column using fluorescence detection and authentic biopterin as a standard. The amount of BH$_4$ in the arteriolar extracts was determined from the difference between acidic and basic iodine-generated biopterin (Meininger *et al.* 2000). The sensitivity of L-arginine and BH$_4$ analyses by HPLC, which was assessed using detection limits defined as a signal-to-noise ratio of 3, was 5 and 2 μM, respectively. The reliability of the assays was indicated by the precision (agreement between replicate measurements), evaluated by the relative deviation (mean of absolute deviation/mean of replicate measurements × 100%), and by the accuracy (the nearness of an experimental value to the true value), determined with known amounts of standards and expressed as the relative errors ((measurement value − true value)/true value × 100%). The precision and accuracy for the L-arginine analysis were 1.4% and 1.6%, respectively, and for the BH$_4$ analysis were 2.0% and 2.3%, respectively. The values in fmol (mg tissue)$^{-1}$ and pmol (mg tissue)$^{-1}$ for BH$_4$ and arginine concentrations, respectively, were calculated on the basis of tissue weight.

Data analysis

Data are expressed as means ± standard error of the mean. Spontaneous tone was calculated as a percent constriction in relation to maximal diameter as determined by the following equation:

$$\text{Spontaneous Tone (\%)} = [(D_M - D_T)/D_M] \times 100$$

where D_M is the maximal diameter recorded at 70 cmH$_2$O and D_T is the steady-state baseline diameter recorded at the same pressure. The vasodilator responses to flow are expressed as percent relaxation as calculated by the formula:

$$\text{Relaxation (\%)} = [(D_S - D_B)/(D_M - D_B)] \times 100$$

where D_S is the arteriolar diameter at each respective stage, D_B is the diameter recorded immediately prior to initiation of the flow–diameter curves, and D_M is the maximal diameter for the arteriole. For statistical analyses, changes in diameter in response to flow were expressed as a percentage of maximal vasodilatation as previously described (Muller-Delp *et al.* 2002). Flow–diameter curves were evaluated by a three-way ANOVA with repeated measures on one factor in order to detect differences within (flow rate) and between (animal groups) factors. Two-way ANOVA was used to determine group differences in animal and vessel characteristics, and group differences in L-arginine and BH$_4$ content in soleus muscle arterioles. Three-way ANOVA was used to determine the effect of age, training and L-NAME treatment on DAF fluorescence. Three-way ANOVA was used to determine the effect of age, training, and L-NAME treatment on DHE fluorescence, and planned contrasts were used as *post hoc* analysis to compare treatment combinations of interest. All data are presented as means ± S.E.M. In all statistical analyses, *n* indicates the number of animals in each group. Significance was defined as $P \leq 0.05$.

Results

Animals

Body mass increased with age and exercise training reduced body mass in both young and old rats (Table 1). Soleus muscle mass increased with age, but was unaltered by exercise training (Table 1). In contrast, soleus muscle mass to body mass ratio decreased with age and increased with exercise training (Table 1). Exercise training increased citrate synthase activity by 18.3% in soleus muscles of young rats, and by 20.1% in soleus muscles of old rats, confirming the efficacy of the exercise training as previously reported (Spier *et al.* 2004).

Maximal intraluminal diameter and levels of spontaneous tone of soleus muscle arterioles were not different among groups (Table 1). Treatment with Tempol, Tempol plus catalase, catalase alone, apocynin and Tempol plus deferoxamine had no effect on spontaneous tone among groups (Table 1).

Table 1. Soleus muscle and vessel characteristics of young and old, sedentary and exercise trained rats

	Young SED ($n = 30$)	Young ET ($n = 37$)	Old SED ($n = 32$)	Old ET ($n = 26$)
Animal characteristics body wt (g)	371 ± 5	348 ± 4	$437 \pm 6*$	$379 \pm 5\dagger$
Soleus muscle wt (mg)	156 ± 4	165 ± 3	$166 \pm 3*$	169 ± 4
Soleus wt/body wt (mg g^{-1})	0.41 ± 0.01	$0.47 \pm 0.01*$	$0.37 \pm 0.01*$	$0.45 \pm 0.01\dagger$
Vessel characteristics maximal diameter (μm)	130 ± 4	127 ± 4	123 ± 4	124 ± 5
Spontaneous tone (%)	51 ± 2	54 ± 2	51 ± 2	57 ± 2
	($n = 30$)	($n = 37$)	($n = 33$)	($n = 26$)
With Tempol	48 ± 5	47 ± 5	61 ± 6	59 ± 3
	($n = 7$)	($n = 8$)	($n = 7$)	($n = 10$)
With Tempol + catalase	50 ± 5	53 ± 4	54 ± 3	57 ± 3
	($n = 5$)	($n = 10$)	($n = 10$)	($n = 7$)
With catalase	45 ± 2	53 ± 5	55 ± 3	56 ± 6
	($n = 6$)	($n = 9$)	($n = 7$)	($n = 9$)
With apocynin	46 ± 3	52 ± 5	57 ± 4	56 ± 4
	($n = 6$)	($n = 6$)	($n = 7$)	($n = 6$)
With Tempol + deferoxamine	54 ± 4	48 ± 5	64 ± 3	60 ± 5
	($n = 6$)	($n = 9$)	($n = 7$)	($n = 3$)

Maximal diameter was recorded in Ca^{2+}-free physiological saline solution with 100 μM sodium nitroprusside. Tone (%) = [(maximal diameter − diameter with tone)/maximal diameter] × 100. *$P < 0.05$ *vs.* young SED and $\dagger P < 0.05$ *vs.* old SED.

Vasodilator responses to flow

Vasodilatation to intraluminal flow was diminished in soleus muscle arterioles from old SED rats (Fig. 1). Exercise training restored flow-induced vasodilatation in soleus muscle arterioles from old rats to that of young SED rats and exercise training improved flow-induced dilatation in soleus muscle arterioles from young rats (Fig. 1).

Arteriolar BH$_4$ and L-arginine content

Similar to previous findings (Delp *et al.* 2008), age decreased arteriolar BH$_4$ levels (Fig. 2A). Exercise training

Figure 2. BH$_4$ and L-arginine levels in soleus muscle arterioles
A, exercise training reverses age-related declines in arteriolar BH$_4$ levels in soleus muscle arterioles. B, ageing and exercise training had no effect on arteriolar L-arginine levels in soleus muscle arterioles. *$P < 0.05$ *vs.* young SED and $\dagger P < 0.05$ *vs.* old SED. $n = 6$–8 per group.
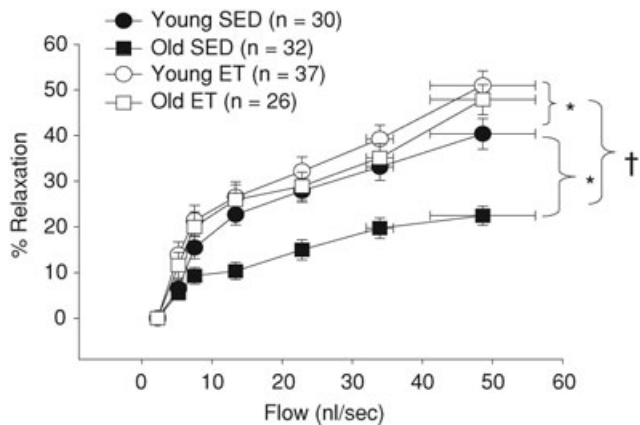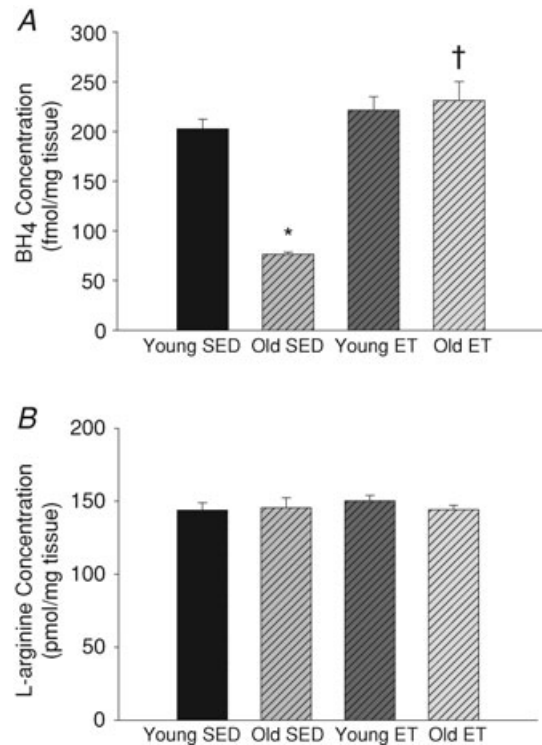
Figure 1. Diameter changes in response to increasing intraluminal flow in soleus muscle arterioles
Exercise training restores flow-induced vasodilatation in arterioles from old rats and improves flow-induced vasodilatation in arterioles from young rats. *$P < 0.05$ *vs.* young SED and $\dagger P < 0.05$ *vs.* old SED.

restored arteriolar BH_4 levels of old rats to that of young rats (Fig. 2*A*). Exercise training did not alter BH_4 levels in soleus muscle arterioles from young rats. As previously reported (Delp *et al.* 2008), ageing had no effect on L-arginine levels and exercise training did not effect arteriolar L-arginine levels in either young or old rats (Fig. 2*B*).

Flow-stimulated NO availability in soleus muscle arterioles

Exposure to intraluminal flow increased DAF fluorescence by 10% above baseline values in arterioles from young SED rats, and by approximately 5% in arterioles from old SED rats (Fig. 3*B*). In arterioles from young ET and old ET rats, DAF fluorescence increased by approximately 23%

and 14% above baseline, respectively, during exposure to flow. L-NAME eliminated flow-stimulated increases in DAF fluorescence in arterioles from all groups of rats, confirming DAF specificity for NO (Fig. 3*B*). Exposure to Dea-NONOate ($10 \mu M$) elicited maximal DAF fluorescence that was similar in arterioles from all groups (young SED, 2.27 ± 0.30; old SED 2.17 ± 0.23; young ET, 2.17 ± 0.27; old ET, 2.23 ± 0.20 fluorescence units), indicating that the dye was not saturated, and that loading of dye did not differ between groups.

Flow-stimulated O_2^- generation in soleus muscle arterioles

Flow-stimulated increases in DHE fluorescence were eliminated by treatment with Tempol, confirming
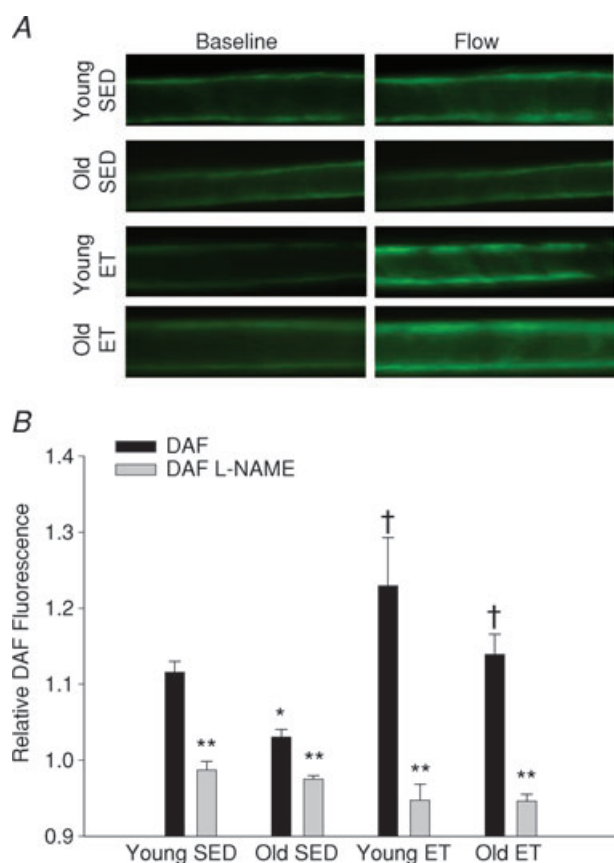

Figure 3. Flow-induced stimulation of arteriolar NO
A, DAF fluorescence indicates NO bioavailability in soleus muscle arterioles from young and old, sedentary and exercise trained rats at baseline and during flow stimulation (34 nl s^{-1}). *B*, flow-stimulated changes in NO bioavailability, shown here as flow-induced to baseline fluorescence ratio (relative fluorescence), is reduced in soleus muscle arterioles from old SED rats. Exercise training restored flow-stimulated NO availability in arterioles from old rats and increased flow-stimulated NO availability in young rats. L-NAME inhibited flow-induced increases in DAF fluorescence in soleus muscle arterioles from all rats. *$P < 0.05$ *vs.* young SED, †$P < 0.05$ *vs.* respective SED group, and **$P < 0.05$ control *vs.* L-NAME. $n = 8–10$ per group.
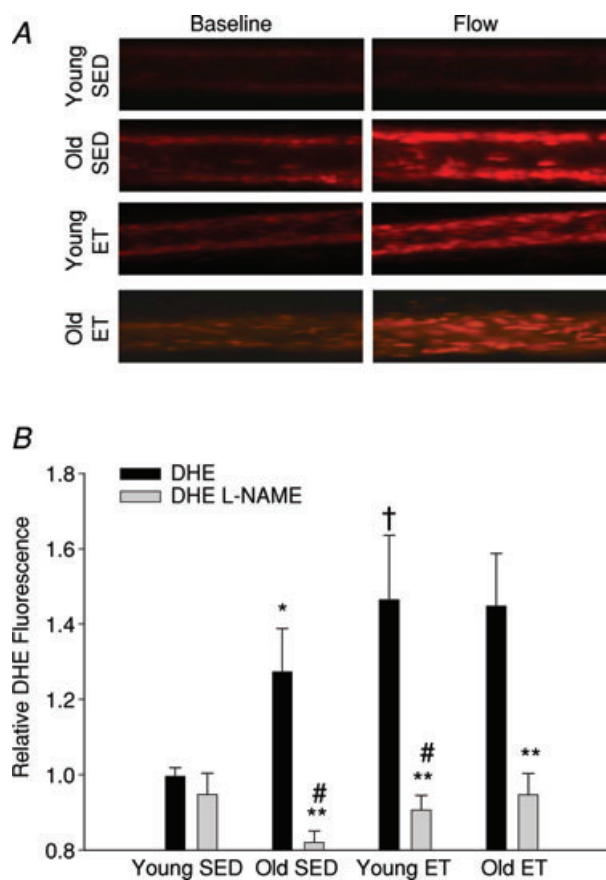
Figure 4. Flow-induced stimulation of arteriolar O_2^-
A, DHE fluorescence indicates O_2^- levels in soleus muscle arterioles from young and old, sedentary and exercise trained rats at baseline and during flow stimulation (34 nl s^{-1}). *B*, flow-stimulated changes in O_2^- levels, indicated here as flow-induced to baseline fluorescence ratio (relative fluorescence), is increased in soleus muscle arterioles from old SED rats. Exercise training increased relative DHE fluorescence in young rats, but had no effect in old rats. L-NAME reduced DHE fluorescence significantly in soleus muscle arterioles from all but young SED rats. *$P < 0.05$ *vs.* young SED, †$P < 0.05$ *vs.* old SED, **$P < 0.05$ control *vs.* L-NAME, and #$P < 0.05$ change in DHE fluorescence with L-NAME *vs.* young SED. $n = 9–11$ per group.

O_2^--specific oxidation of DHE (data not shown). The relative increase in DHE fluorescence that occurred in response to exposure to intraluminal flow was greater in soleus muscle arterioles from old SED rats as compared to those from young SED rats (Fig. 4*B*). Exercise training augmented the relative increase in O_2^- produced by intraluminal flow exposure in arterioles of young rats, but did not alter the relative amount of O_2^- generated by flow stimulation in arterioles from old rats, indicating that augmented O_2^- signalling contributes to exercise training-induced improvement of flow-induced vasodilatation in young rats. Inhibition of eNOS with L-NAME reduced the flow-induced DHE fluorescence signal significantly in arterioles from all but young SED rats (Fig. 4*B*), implicating eNOS as a significant source of flow-induced O_2^- generation. L-NAME inhibition of flow-induced DHE fluorescence was significant in arterioles from old SED rats but not in arterioles from young SED rats, suggesting that flow stimulation of eNOS-derived O_2^- increases with age. L-NAME inhibited flow-induced increases in DHE fluorescence in arterioles from both young and old ET rats, suggesting that exercise training increases signalling through eNOS-derived O_2^- in arterioles from rats of both ages. Exposure to ethanol induced maximal DHE fluorescence that was not different between groups (young SED, 315 ± 0.50; old SED 290 ± 0.29; young ET, 296 ± 0.41; old ET, 315 ± 0.35 fluorescence units), confirming that loading of dye was similar in arterioles from all groups of rats.

Role of O_2^- and H_2O_2 and in flow-induced vasodilatation

In young SED rats, scavenging of O_2^- with Tempol did not alter flow-induced vasodilatation (Fig. 5*A*). Catalase
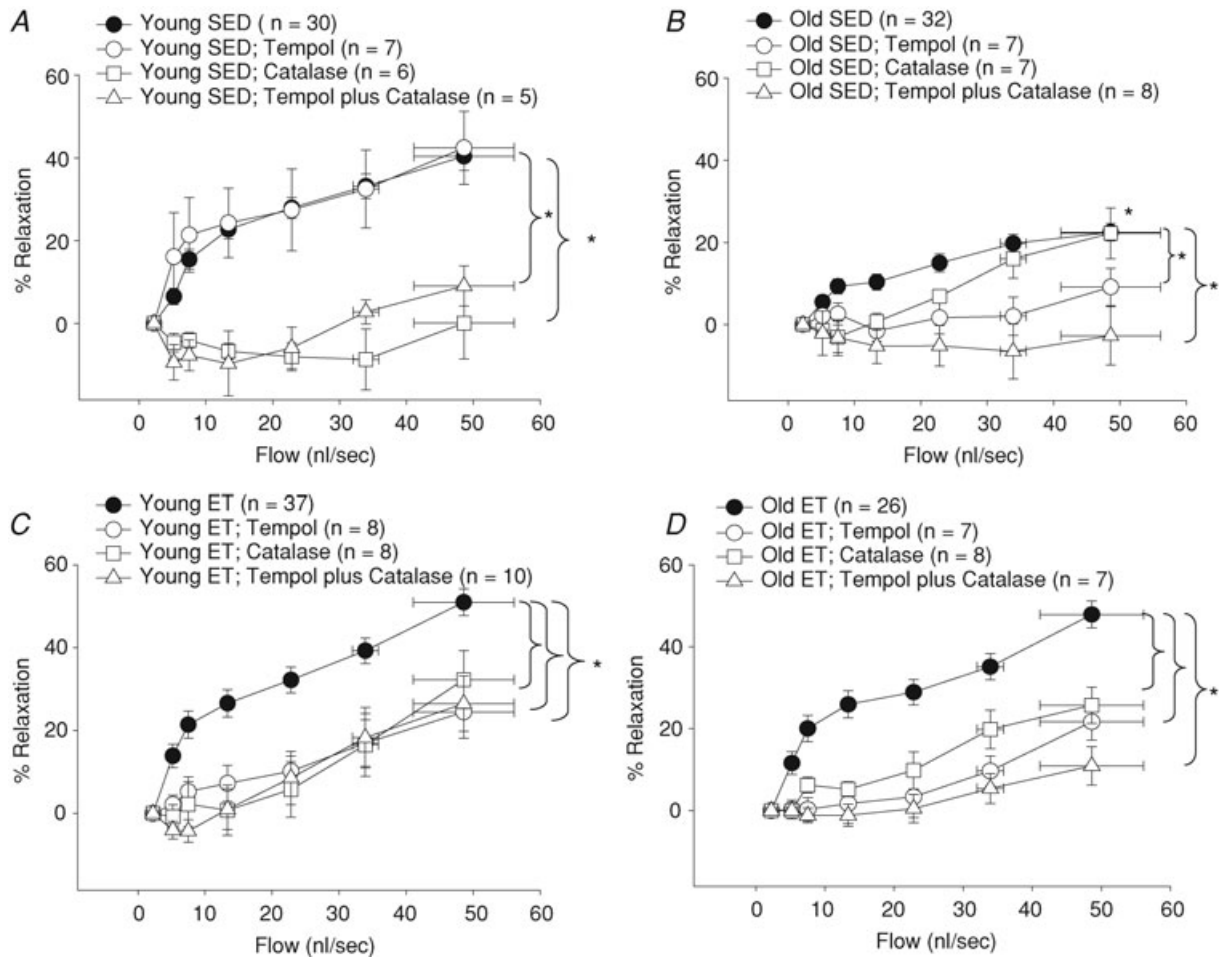

Figure 5. Effects of Tempol and catalase on flow-induced vasodilatation of soleus muscle arterioles from young and old, SED and ET rats
A, Tempol had no effect, but catalase and Tempol + catalase completely abolished flow induced vasodilatation in young SED. *B*, in old SED rats, catalase, Tempol, and Tempol + catalase reduced flow-induced vasodilatation. *C*, all treatments reduced flow-induced vasodilatation in young ET and (D) old ET rats. *$P < 0.05$ *vs.* control response.

and Tempol plus catalase inhibited flow-induced vaso-dilatation indicating dependence on H_2O_2 in young SED rats (Fig. 5*A*).

Scavenging of O_2^- with Tempol inhibited flow-induced vasodilatation in arterioles from old SED rats (Fig. 5*B*). Scavenging of H_2O_2 with catalase reduced flow-induced vasodilatation between flow rates of 5.2 ± 0.75 and 22.8 ± 0.42 nl s^{-1} but not at higher flow rates, and Tempol plus catalase eliminated flow-induced vasodilatation in the old SED rats, suggesting that despite an overall reduction of flow-induced dilatation, H_2O_2 functions as a significant vasodilator in arterioles from old SED rats (Fig. 5*B*).

In young and old ET rats, scavenging of O_2^- with Tempol reduced flow-induced vasodilatation (Fig. 5*C* and *D*). Likewise, the scavenging of H_2O_2 with catalase alone reduced flow-induced dilatation in arterioles from young and old ET rats (Fig. 5*C* and *D*). Simultaneous scavenging of both O_2^- and H_2O_2 with Tempol plus catalase nearly abolished flow-induced vasodilatation in arterioles from old ET rats (Fig. 5*D*). The lack of a significant residual flow-induced vasodilatation following simultaneous scavenging of O_2^- and H_2O_2 (Fig. 5*D*) indicates that H_2O_2 is critical for flow-induced vaso-dilatation in arterioles from old ET rats. In contrast, although simultaneous scavenging of O_2^- and H_2O_2 reduced flow-induced vasodilatation in arterioles from young ET rats (Fig. 5*C*), significant dilatation remained in the presence of these scavengers, suggesting that NO contributes substantially to flow-induced dilatation in arterioles from these rats.

The inhibition of NADPH oxidase with apocynin reduced flow-induced vasodilatation in young and old SED rats (Fig. 6*A*). However, apocynin had no effect on flow-induced vasodilatation in young and old ET rats (Fig. 6*B*), suggesting that O_2^--derived H_2O_2 required for flow-induced vasodilatation in the ET rats is not generated from NADPH oxidase-derived O_2^-.

Role of OH^- in flow-induced vasodilatation

SOD has the potential to generate OH^- in the presence of iron, and OH^- may cause vasoconstriction (Bulvik *et al.* 2008). The addition of deferoxamine, an iron chelator, to Tempol-treated arterioles had no effect in young SED rats (Fig. 7*A*); however, iron chelation restored the Tempol-induced loss of flow-induced vasodilatation in arterioles from old SED (Fig. 7*B*) suggesting that OH^- generation in Tempol-treated arterioles elicits vasoconstriction. Deferoxamine added to Tempol-treated arterioles improved the Tempol-induced loss of flow-induced vasodilatation observed in young and old ET rats (Fig. 7*C* and *D*), confirming that O_2^- production increases with exercise training and may contribute to increased OH^--induced vasoconstriction in the presence of exogenous SOD and sufficient free iron.

Discussion

Several important new findings emerge from this study. First, the balance between NO and O_2^- signalling is
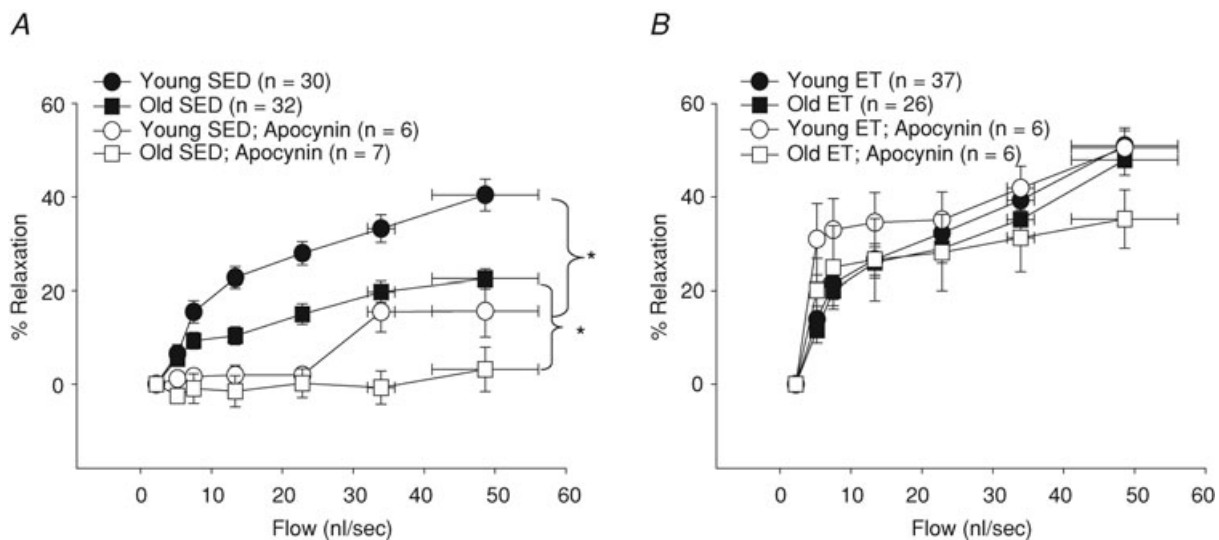

Figure 6. Role of NADPH oxidase-derived O_2^- production in flow-induced vasodilatation in soleus muscle arterioles of young and old, SED and ET rats
A, NADPH oxidase inhibition with Apocynin reduced flow-induced vasodilatation in soleus muscle arterioles of young and old SED rats. *B*, inhibition of NADPH oxidase had no effect on flow-induced vasodilatation in soleus muscle arterioles of young and old ET rats. *$P < 0.05$ *vs.* control response.

altered by age and exercise training in skeletal muscle arterioles. Second, BH_4 content is reduced with age and restored with exercise training in skeletal muscle arterioles from old rats, whereas neither ageing nor training altered arteriolar arginine levels. The age-related decline in arteriolar BH_4 content is concomitant to a decrease in NO signalling and an increase in ROS signalling. Further, the exercise-induced restoration of BH_4 content coincides with an increased NO signalling in skeletal muscle arterioles of old ET rats. Third, under conditions of limited BH_4, such as occurs with old age, NO-mediated signalling declines and eNOS-generated ROS (O_2^-) increases in skeletal muscle arterioles. And finally, exercise training increases both NO- and ROS-mediated signalling in skeletal muscle arterioles of young rats, but a balance in

these signalling systems is maintained, resulting in robust flow-induced vasodilatation.

Ageing is associated with declines in flow-induced NO-dependent vasodilatation in conduit and resistance arterioles of humans and rats (Gerhard *et al.* 1996; DeSouza *et al.* 2000; Muller-Delp *et al.* 2002; Woodman *et al.* 2002). Limited NO bioavailability may be mediated by several possible mechanisms, including reduced eNOS protein abundance or activity, limited availability of substrate (L-arginine) or co-factor (BH_4), or increased degradation of NO by ROS. We recently reported that a deficit in availability of BH_4 is associated with an age-dependent decline of endothelium-dependent vasodilatation in skeletal muscle arterioles, and that supplementation with the BH_4 precursor sepiapterin
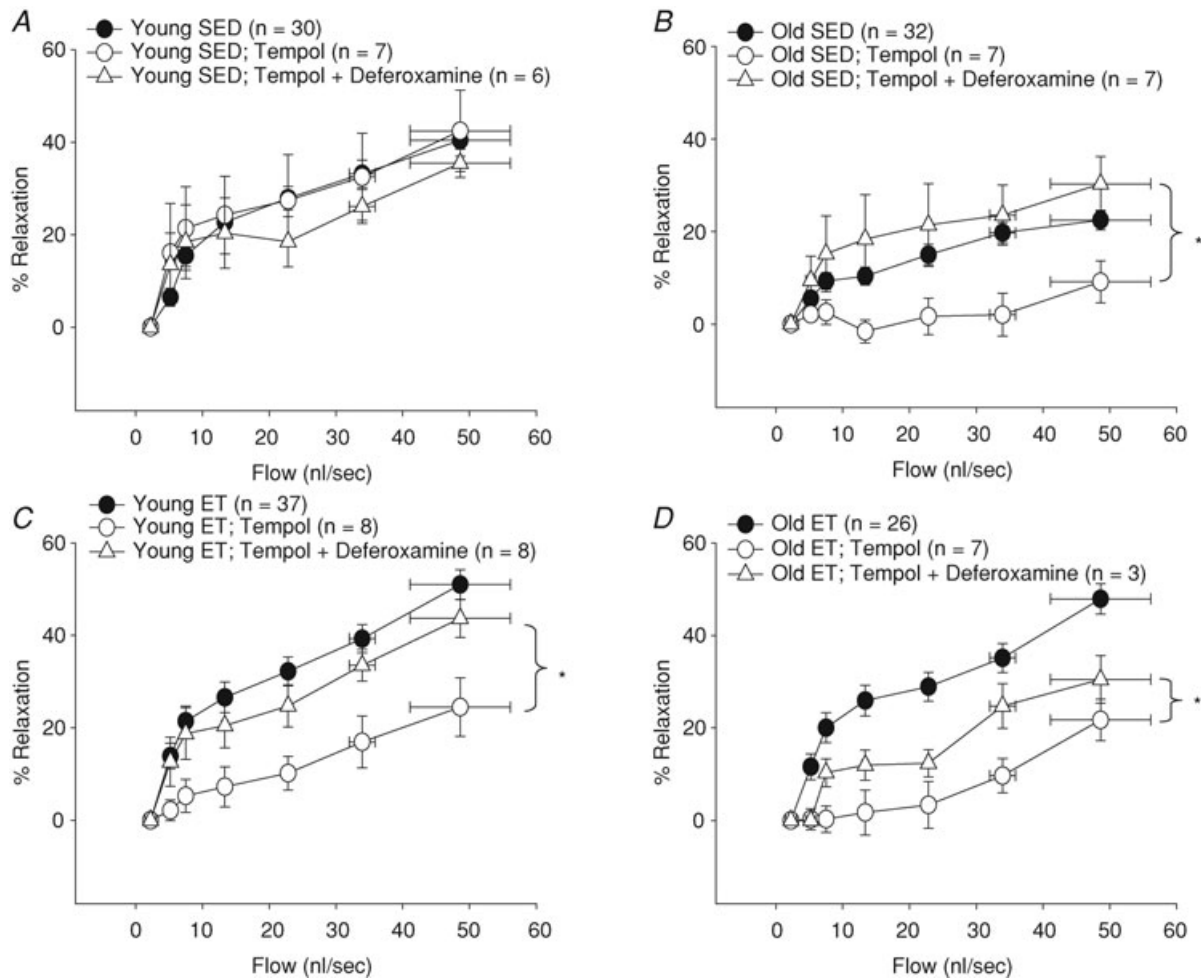

Figure 7. Effects of Tempol and deferoxamine on flow-induced vasodilatation in soleus muscle arterioles of young and old, SED and ET rats

A, neither Tempol or deferoxamine, an iron chelator, reduced flow-induced vasodilatation in young SED. *B*, Tempol reduced and Tempol + deferoxamine improved flow-induced vasodilatation in old SED. *C*, Tempol reduced and Tempol + deferoxamine restored flow-induced vasodilatation in young ET. *D*, Tempol reduced flow-induced vasodilatation; however, Tempol + deferoxamine only partially restored flow-induced vasodilatation in the old ET. $*P < 0.05$ *vs.* control response.

partially restores flow-induced, endothelium-dependent vasodilatation in skeletal muscle arterioles from old rats (Delp *et al.* 2008). Eskurza *et al.* (2005) reported that an acute bolus of BH_4 restored flow-mediated dilatation in older sedentary humans. These findings support the hypothesis that the old age-associated decrements in BH_4 contribute to reduced NO bioavailability (Fig. 8). When BH_4 is limited, eNOS has been shown to become uncoupled, producing both O_2^- and NO (Bevers *et al.* 2006; Crabtree *et al.* 2009); thus, the current study was designed to test the hypothesis that eNOS uncoupling increases with age in skeletal muscle arterioles. To our knowledge, this is the first study to demonstrate that an old age-related reduction of BH_4 (Fig. 2*A*) is accompanied by impaired flow-induced NO signalling (Fig. 3) and increased O_2^- signalling in skeletal muscle arterioles (Fig. 8). Although DHE can be oxidized by various ROS including NO, our fluorescence data also suggest that the age-associated decrement in BH_4 availability contributes to uncoupling of eNOS and an increase in eNOS-derived O_2^- generaton in response to flow stimulation. Further study is required to determine whether augmentation of endothelial BH_4 can reverse eNOS uncoupling and restore NO signalling in arterioles from old rats.

Increases in O_2^- generation, observed with age, scavenge NO and may alter endothelial function (Donato *et al.* 2007). In soleus muscle feed arteries, SOD-1 protein content, but not mRNA expression, is reduced in aged rats (Woodman *et al.* 2002); however, SOD-1 activity appears to increase in the aorta of aged rats (Demaree *et al.* 1999). Additionally, catalase activity declines in aorta from aged rats (Demaree *et al.* 1999); however, catalase protein content does not change with age in human endothelial cells (Donato *et al.* 2007). Exercise training potentially improves NO bioavailability by enhanced regulation of O_2^- and ROS by inducing antioxidant defence systems and reducing O_2^- generating
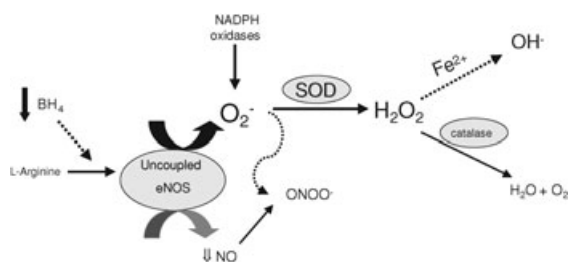

Figure 8. The proposed effects of ageing on eNOS uncoupling
BH_4 content is reduced in soleus muscle arterioles of aged rats. With limited availability of BH_4, eNOS becomes uncoupled and leads to generation of O_2^- rather than NO. Peroxynitrite ($ONOO^-$), a product of NO and O_2^- generated through eNOS uncoupling, NADPH oxidases and other sources, could further reduce BH_4 availability. Vascular SOD scavenges O_2^-, which can produce the vasodilator H_2O_2. In the presence of catalytic transition metals (i.e. Fe^{2+}), H_2O_2 can produce the potent vasoconstrictor OH^-. H_2O_2 is converted to H_2O and O_2 in the presence of catalase.

systems. Rush *et al.* (2000) demonstrated in aortic endothelial cells (AEC) from young pigs that exercise training increased SOD-1 protein abundance and activity but had no effect on catalase content. In contrast, exercise training decreased the NADPH pro-oxidant subunit, p67phox protein. Graham & Rush (2004) further demonstrated that the cytosolic subunit of NADPH oxidase, gp91phox, is reduced with training in aorta homogenates from young rats. Based on these observations, it appears that exercise training improves the management of superoxide (and subsequent ROS) generation by enhancing anti-oxidant mechanisms and reducing pro-oxidant pathways. However, a paradox emerges as O_2^- generation and oxidant stress increases with exercise training (Davies *et al.* 1982; Alessio, 1993; Alessio *et al.* 2000). Our data suggest that exercise training-induced enhancement of flow-induced vasodilatation occurs because both NO- and ROS-mediated signalling increase in a balanced fashion.

Even though high levels of ROS may potentiate the pathology of cardiovascular diseases (Harrison, 1997), it is becoming increasingly clear that ROS can serve as signalling molecules, which mediate normal cellular functions (Droge, 2002). H_2O_2 generated by the dismutation of O_2^- is emerging as an important vasodilator and signalling molecule in the vasculature. Endothelium-derived H_2O_2 contributes to flow-induced vasodilatation in isolated coronary arterioles (Koller & Bagi, 2004). Marvar *et al.* (2007) demonstrated that H_2O_2 produced by contracting skeletal muscle elicits a vasodilatory response on nearby skeletal muscle arterioles. Furthermore, exogenous H_2O_2 is vasodilatory in a variety of vascular beds including pulmonary (Burke & Wolin, 1987), skeletal muscle (Cseko *et al.* 2004), cerebral (Wei & Kontos, 1990) and mesenteric beds (Matoba & Shimokawa, 2003). H_2O_2 also indirectly alters vascular reactivity by activating eNOS via phosphoinositide 3-kinase (PI3K) (Thomas *et al.* 2002). Our data indicate that H_2O_2 is an important mediator of flow-induced vasodilatation of skeletal muscle arterioles from young SED rats. In addition, our data suggest that flow-induced H_2O_2 signalling declines with age, but is restored by exercise training.

SOD is an important anti-oxidant enzyme that reduces oxidant stress by dismutating O_2^- into H_2O_2; however, in the presence of catalytic transition metals, SOD can rapidly form OH^- (Pattwell *et al.* 2004). H_2O_2 generates OH^- through metal-catalysed reactions, such as the Fenton reaction as follows: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^\bullet OH + OH^-$. The formation of OH^- is further promoted by the presence of O_2^-, which reacts with Fe^{3+} to produce Fe^{2+} through the Haber–Weiss reaction (Halliwell, 1989; Powers & Jackson, 2008). The net effect of SOD is the dismutation of O_2^- to produce either the vasodilatory H_2O_2 or in the presence of Fe^{2+}, OH^-, which is a potent vasoconstrictor (Fig. 8). Iron

chelators, such as deferoxamine, inhibit the generation of OH^- by preventing iron ions from catalysing redox reactions, thereby improving vasodilatation in coronary arterioles (Nitenberg *et al.* 1998; Thengchaisri *et al.* 2006). Iron accumulation increases with age in skeletal muscle, liver and cardiac muscle, and is associated with increased oxidative stress and diminished functional capacity (Bulvik *et al.* 2008; Xu *et al.* 2008). Old age-associated increases in iron accompanied by diminished catalase activity (Demaree *et al.* 1999) could produce excess OH^- contributing to decreased flow-induced vasodilatation in skeletal muscle arterioles of old rats (Fig. 2*B*). Exercise training increases iron in skeletal muscle of rats (Navas & Cordova, 2000) and catalase activity in the vasculature remains unchanged with exercise training (Rush *et al.* 2000). Our data indicate that exercise training increases flow-induced O_2^- generation in arterioles from young and old rats. Therefore, it seems plausible that this increase in flow-induced O_2^- generation could also result in elevated OH^- production in arterioles treated with an exogenous SOD mimetic. In addition, our data indicate that iron chelation by deferoxamine improves flow-induced vasodilatation in skeletal muscle arterioles treated with the SOD mimetic Tempol, suggesting that inhibition of OH^- production reverses the Tempol-induced reduction of flow-induced vasodilatation.

In conclusion, the results of the present study confirm previous findings that ageing impairs endothelium-dependent, NO-mediated vasodilatation and BH_4 levels in rat skeletal muscle arterioles (Muller-Delp *et al.* 2002; Delp *et al.* 2008). The present findings further demonstrate that limited availability of BH_4, as observed in old SED rats, may contribute to eNOS uncoupling, and a subsequent decrease in NO signalling and an increase in eNOS-derived O_2^- generation as illustrated in Fig. 8. Exercise training restores BH_4 levels and improves flow-stimulated NO availability in arterioles from aged rats. Furthermore, exercise training increases both NO and ROS-mediated signalling in skeletal muscle arterioles, suggesting that exercise training-induced enhancement of flow-induced vasodilatation in skeletal muscle arterioles involves a balance between NO and O_2^--derived ROS. Our results suggest that the beneficial effects of exercise training in the skeletal muscle resistance vasculature involve much more than improvement of NO bioavailability.

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Author contributions

A.L.S. contributed to experimental design, analysis and interpretation, and writing of this article. M.D.D. contributed to experimental design, data interpretation, and critical revision of this article. R.R. contributed to data collection, data analysis and interpretation, and critical revision of this article. G.W. contributed to experimental design, data analysis and interpretation, and critical revision of this article. J.M.D. contributed to conception and design of experiments, data interpretation, and writing of this article.

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