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Effects of aging and exercise training on eNOS uncoupling and reactive oxygen species signaling in the endothelium of skeletal muscle arterioles

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Effects of aging and exercise training on eNOS uncoupling and reactive oxygen species signaling in the endothelium of skeletal muscle arterioles

Amy L. Sindler

**Dissertation submitted to the
School of Medicine
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy
in
Cellular and Integrative Physiology**

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Abstract

Effects of aging and exercise training on eNOS uncoupling and reactive oxygen species signaling in the endothelium of skeletal muscle arterioles

Amy L. Sindler

The purpose of the first study was to determine the effects of aging and exercise training on endothelial nitric oxide synthase (eNOS) uncoupling in skeletal muscle arterioles. The results of this study confirmed our previous findings that aging impairs endothelium-dependent, NO-mediated vasodilation and tetrahydrobiopterin (BH₄) levels in rat skeletal muscle arterioles. Limited availability of BH₄, as observed in old sedentary rats, may contribute to eNOS uncoupling, which decreases NO signaling and increases eNOS-derived O₂⁻ generation. Exercise training restored BH₄ levels and improved flow-induced NO production in arterioles from aged rats. Furthermore, exercise training increased both NO and reactive oxygen species (ROS)-mediated signaling in skeletal muscle arterioles, suggesting that exercise training-induced enhancement of flow-induced vasodilation in skeletal muscle arterioles involves a balance between NO and O₂⁻-derived ROS.

The second study determined the role of O₂⁻-derived ROS in mediating endothelium-dependent vasodilation in skeletal muscle arterioles from young and old, sedentary and exercise trained rats. The results of the second study implicated O₂⁻ and O₂⁻-derived ROS (H₂O₂) as necessary signaling molecules required for endothelium-dependent vasodilation in soleus muscle arterioles. The dependence of ACh-induced vasodilation on H₂O₂ increased with age and decreased with exercise training. Exercise training contributed to appropriate regulation of the relative production of O₂⁻ and H₂O₂, which must be maintained for robust endothelium-dependent vasodilation to occur in skeletal muscle arterioles.

Preface

This dissertation will begin with an introduction that constitutes the scientific background for which these studies were conducted. The two studies that comprise this dissertation work will be reported in manuscript form, followed by a general discussion. This dissertation follows the style and format of the *Journal of Applied Physiology*.

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Glossary of Abbreviations

ACh	acetylcholine
ADMA	asymmetric dimethylarginine
AEC	aortic endothelial cells
A-VO ₂ Δ	arteriovenous O ₂ difference
BH ₃	trihydrobiopterin radical
BH ₄	tetrahydrobiopterin
BW	body weight
cAMP	3',5'-cyclic adenosine monophosphate
cGMP	3'5'-cyclic guanosine monophosphate
CVD	cardiovascular disease
D _m	maximal diameter
D _s	steady state diameter measured after intervention
D _t	steady state baseline diameter
DAF-2DA	4,5-diaminofluorescein diacetate
Dea-NONOate	diethylamineNONOate
DHE	dihydroethidium
EDHF	endothelial derived hyperpolarizing factor
eNOS	endothelial nitric oxide synthase
ET	exercise trained
H ₂ O	water
H ₂ O ₂	hydrogen peroxide

HPLC	high-performance liquid chromatography
GPx	glutathione peroxidase
GTP	guanosine-5'-triphosphate
GTPCH-1	GTP cyclohydrolase
K _{ATP} channel	ATP-sensitive potassium channel
K _{Ca} channel	calcium-activated potassium channel
L-NAME	N ^G -nitro-L-arginine methyl ester
NADPH	nicotinamide adenine dinucleotide phosphate
NIH	National Institutes of Health
NOHA	N ^ω -hydroxy-nor-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
O ₂ ⁻	superoxide anion
OH ⁻	hydroxyl anion
ONOO ⁻	peroxynitrite
PSS	physiological saline solution
Q	flow
ROS	reactive oxygen species
sGC	soluble guanylate cyclase
SOD	superoxide dismutase
SNP	sodium nitroprusside
VSM	vascular smooth muscle

V_{rbc}	mean red cell velocity
$\text{VO}_{2 \text{ max}}$	maximal oxygen consumption
WVU	West Virginia University

Chapter I

Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States claiming over 36% of all deaths per year. In 2005, the Center for Disease Control estimated that over 80 million people suffered from some form of CVD and the risk for developing CVD increases with advancing age (1). In 2007, the US Census Bureau estimated that over 42 million individuals currently living in the US are 65 years of age or older. This number is estimated to double by the year 2030 (2). As our population ages, the importance of preventing and/or prolonging the development of CVD becomes a high priority. It has been well established that regular physical activity improves overall health and reduces risk factors for many chronic diseases (i.e., obesity, diabetes, hypertension, and certain cancers). However, the mechanisms by which exercise improve cardiovascular health remains largely unknown.

Age-related declines in exercise capacity due to cardiovascular adaptations

Maximal exercise capacity declines with advancing age (47, 104) and this is due, in part, to systemic cardiovascular adaptations. For example, maximal oxygen consumption ($\text{VO}_{2\text{ max}}$) decreases 5-15% every decade of life after the age of 25 (63). Factors that contribute to age-associated declines in $\text{VO}_{2\text{ max}}$ are decreases in maximal cardiac output and maximal arteriovenous O_2 difference ($\text{A-VO}_2 \Delta$) (104, 112, 125). Other factors that contribute to age-associated declines in $\text{VO}_{2\text{ max}}$ include decreases in maximal heart rate and stroke volume, (112, 125) decreases in skeletal muscle mass, and increases in adiposity (104). Although systemic cardiovascular adaptations markedly influence exercise capacity in older individuals, it is possible that age-associated dysregulation of skeletal muscle blood flow limits exercise capacity in older individuals. Work in humans and animals indicates that blood flow to actively contracting muscles declines with age (35, 67). Proctor et al. (109) demonstrated that leg blood flow and

vascular conductance were reduced in older males (55-68 yr) during exercise compared to young males (22-30 yr). More importantly, age alters the distribution of muscle blood flow in rats by redistributing blood flow away from highly oxidative muscles and to highly anaerobic muscles (98). Age-associated decrements in skeletal muscle blood flow during exercise may contribute to overall diminution in exercise capacity; however, little is known about age-related alterations of vascular control mechanisms in skeletal muscle.

Skeletal muscle blood flow is tightly controlled by the resistance vasculature and is dependent on a functioning endothelium. The endothelium, which lines the interior surface of blood vessels, plays an important role in maintenance of vascular tone and control of vascular resistance (65). Endothelial cells produce nitric oxide (NO) synthesized from L-arginine by endothelial nitric oxide synthase (eNOS). NO is a critical signaling molecule in vascular homeostasis. NO acts as an antiatherosclerotic molecule by inhibiting vascular smooth muscle proliferation, platelet aggregation, and leukocyte adhesion (65). Additionally, NO produced by endothelial cells rapidly diffuses to vascular smooth muscle (VSM) cells, where it activates cyclic guanosine monophosphate (cGMP) and causes vasodilation.

Age-related declines in NO-mediated endothelial-dependent vasodilation and reversal with exercise training

Decreased NO bioavailability is a consequence of aging and contributes to impaired endothelial function (17). Impaired NO-mediated endothelial function serves as both a predictor and mediator of CVD. Endothelial dysfunction is thought to be the critical determinant in development of atherosclerosis (17, 18, 78). Many studies have demonstrated the age-associated loss of endothelium-dependent vasodilation in skeletal muscle conduit and resistance vasculature in humans and animals (38, 54, 97, 136, 145). Age-associated impairments of both flow-induced and receptor-mediated endothelium-dependent vasodilation occur through reduced NO signaling

(122, 123). Aerobic exercise training improves endothelium-dependent, NO-mediated vasodilation in humans (38, 130, 138) and animals (36, 56, 113, 122, 123, 147, 149, 151). Exercise training not only reverses age-related declines in endothelial function, it also prevents the loss of endothelial function in older individuals who remain physically active throughout their lives (38, 129). Although, the benefits of aerobic exercise training on the cardiovascular system have been widely acknowledged, the definitive mechanisms by which exercise training improves endothelium-dependent vasodilation remain unclear. Exercise training increases both eNOS mRNA (117, 122, 144) and protein content in arterioles and endothelial cells from animals (31, 68, 117, 122, 144) suggesting that enhanced NO production contributes to improvement of endothelial function.

Exercise training may also increase NO bioactivity in the vessel wall by enhancing the sensitivity of vascular smooth muscle to NO. In exercise-trained rats, increased protein content of soluble guanylate cyclase (sGC) may contribute to increased NO sensitivity of the vascular smooth muscle (56). In arterioles from gastrocnemius muscle, exercise training increased sensitivity to the NO donor, sodium nitroprusside (87). Conversely, exercise training has no effect on the sensitivity of vascular smooth muscle to NO in pulmonary arteries or in resistance arterioles from skeletal muscle (68, 122).

Age-associated reduction in NO bioavailability

Several mechanisms may contribute to the age-associated decrease in NO bioavailability in skeletal muscle vasculature. NO production is regulated by several factors including: 1) eNOS enzyme activity or protein content, 2) the formation of calcium/calmodulin complexes (49, 116), and 3) availability of the substrate L-arginine and the co-factor tetrahydrobiopterin (BH₄). In the skeletal muscle vasculature of aged rats, eNOS protein is elevated, suggesting that decreased eNOS protein content does not underlie reduced NO availability (122). Acute L-arginine

treatment improves endothelium-dependent vasodilation in people with cardiovascular disease; however, L-arginine does not enhance flow-induced vasodilation in old rats (122) or humans (11, 53). Furthermore, arteriolar L-arginine levels are unaffected by age in both rats and humans (33, 53). L-arginine bioavailability may be limited by the endogenous inhibitor, asymmetric dimethylarginine (ADMA) (22), or by increased degradation by arginase (7, 141); however, in human vascular endothelial cells and rat skeletal muscle arterioles, both ADMA and arginase protein levels (33, 53) do not increase with age. Furthermore, inhibition of arginase activity by N^ω-hydroxy-nor-L-arginine (NOHA) does not improve flow-induced vasodilation in skeletal muscle arterioles of old rats (33). The synthesis of NO is dependent on the availability of BH₄ (24, 135). With sufficient amounts of BH₄ available, eNOS accepts and stores an adequate number of electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to transform L-arginine and O₂ into NO and L-citrulline (69). If the amount of BH₄ is insufficient, eNOS is unable to catalyze the oxidation of L-arginine. Instead, eNOS accepts electrons from NADPH, and donates the electrons one at a time to O₂, resulting in increased O₂⁻ production and decreased NO production (69). It has been reported that BH₄ is reduced in skeletal muscle arterioles of old rats suggesting that limited arteriolar BH₄ contributes to age-related impairment of NO-dependent vasodilation (33).

The synthesis of BH₄ is regulated by two known mechanisms: 1) The *de novo* pathway and 2) the salvage pathway. In the *de novo* pathway, GTP cyclohydrolase (GTPCH-1) cleaves GTP to produce 7,8-dihydroneopterin triphosphate that is converted to 6-pyruvoyl-tetrahydropterin. In the final step requiring NADPH, sepiapterin reductase converts 6-pyruvoyl-tetrahydropterin to BH₄ (134). BH₄ is also synthesized by the salvage pathway, which recycles BH₂ and quinoid-dihydrobiopterin back to BH₄ (19). In the salvage pathway, the BH₄ precursor, sepiapterin, is converted to BH₄ (100). GTPCH-1 is the rate limiting enzyme in *de novo* BH₄

synthesis and stimulation of this enzyme increases BH₄ levels in human endothelial cells and in resistance mesenteric arteries (16, 43). H₂O₂ augments GTPCH-1 protein content and BH₄ synthesis in endothelial cells (118, 119). Shear stress also stimulates GTPCH activity in human endothelial cells (142). Reports of the effects of age on GTPCH-1 protein are limited. In carotid arteries of mice, GTPCH-1 is unaffected by age (10). Preliminary data from our lab indicate that GTPCH-1 content decreases with age in skeletal muscle resistance arteries (unpublished data).

Stimulation of BH₄ synthesis improves NO production in endothelial cells (119), whereas age-induced impairment of endothelial function is accompanied by reduced BH₄ in skeletal muscle arterioles. In aged humans, an acute oral supplementation with BH₄ improved flow-mediated dilation (45). Moreover, BH₄ co-infusion with acetylcholine increased endothelium-dependent vasodilation to a greater extent in elderly subjects compared with acetylcholine infusion alone (64). In skeletal muscle arterioles from old rats, the BH₄ precursor, sepiapterin improved flow-induced vasodilation (33). Together, these reports suggest that limited BH₄ availability contributes to age-related decrements in endothelial function.

BH₄ and eNOS uncoupling

Uncoupled eNOS has the potential to generate considerable O₂⁻ based on the availability of L-arginine and BH₄ (8, 69). O₂⁻ reacts with NO to form cytotoxic peroxynitrite, ONOO⁻. ONOO⁻ can reduce BH₄ to the inactive trihydrobiopterin radical (BH₃) (75). Thus, a vicious cycle ensues in which BH₄ levels are further reduced, and eNOS uncoupling is exacerbated.

One possible mechanism proposed to increase NO bioavailability is to use eNOS gene transfer to increase eNOS protein content and thus eNOS activity. However, in prehypertensive (spontaneously hypertensive) rats, elevation of eNOS protein was accompanied by decreased NO bioavailability and increased O₂⁻ production (25). When elevated levels of eNOS protein are sustained, it is the loss of BH₄, not L-arginine, that exacerbates eNOS uncoupling (8). Similarly,

in skeletal muscle arterioles from aged rats, increased eNOS protein abundance is accompanied by reduced BH₄ levels and diminished NO-mediated vasodilation suggesting that eNOS uncoupling reduces NO bioavailability with age (33, 122).

Role of oxidant stress on NO bioavailability

O₂⁻ reacts readily with NO to form ONOO⁻ (29). O₂⁻ is generated in the vasculature by NADPH oxidase, by xanthine oxidase, and by eNOS (14, 29, 57). Age-associated O₂⁻ production increases in vascular tissue with age and this increase is thought to be a major factor in the loss of NO bioavailability. Antioxidant defense mechanisms are present in vascular cells to sequester and buffer O₂⁻ and other ROS. Superoxide dismutase (SOD) dismutates O₂⁻ to H₂O₂ (91, 132). There are three known isoforms of the enzyme (SOD-1, SOD-2, and SOD-3); two of which are Cu/Zn-dependent. SOD-1 is primarily located in the cytosol and nucleus and SOD-3 is abundant in the extracellular matrix (29, 94). SOD-2 is Mn-dependent and is most abundant in the mitochondria (143). Other ROS defense mechanisms present in the vascular cells include catalase and glutathione peroxidase (GPx), which reduce H₂O₂ to H₂O and O₂. Together, these defense systems regulate ROS levels in the cell. The balance of superoxide production and antioxidant regulation is thought to be critical for preservation of bioavailable NO (29, 70).

Effects of age and exercise training on regulation of vascular ROS

The primary free radicals generated by endothelial cells are O₂⁻ and NO. Free radicals are defined as an atom or molecule that contains one or more unpaired electrons and is capable of independent existence (59). O₂⁻ is generated as an intermediate in many biochemical reactions by the incomplete reduction of O₂ during electron transport (59). NO is generated by three known isoforms of nitric oxide synthase (NOS). O₂⁻ and NO are both highly reactive and quickly form other ROS (107). In the vascular wall, O₂⁻ is generated by NADPH oxidase, by xanthine oxidase, and by eNOS (14, 29, 70, 92). Of these, NADPH oxidases are the major source of O₂⁻ production

in the vasculature (57). Thus, greater O_2^- production is accompanied by decreased NO bioavailability because O_2^- readily inactivates NO.

SOD serves as a cellular antioxidant by dismutating O_2^- to H_2O_2 ; however, in the presence of catalytic transition metals, SOD can produce hydroxyl radicals (OH^\cdot) (107). In compartments containing both NO and SOD, a reaction between O_2^- and NO occurs more readily than a reaction between O_2^- and SOD (55). Thus some reactivity between O_2^- and NO is likely to be present in endothelial cells, even under normal physiological conditions. Basally, O_2^- is required for normal function, serving as a signaling molecule, involved in regulation of gene expression, and force generation in skeletal muscle (41, 108, 110, 111, 120). Low to moderate levels of ROS may also function in vascular signaling. In fact, many ROS, including NO, H_2O_2 , $ONOO^-$, and OH^\cdot exhibit vasoactive properties that alter vascular tone and regulate blood flow (27, 85, 102, 103, 115, 131). The idea that all ROS are detrimental to the vasculature appears to be a misconception. For example, H_2O_2 is an important vasoactive substance and has been labeled as an endothelium-derived hyperpolarizing factor (EDHF) in certain vasculature beds (84, 85, 91). Additionally, H_2O_2 is an important regulator of eNOS protein expression and activity (133, 152). eNOS-dependent O_2^- generation is required to provide the substrate (O_2^-) to produce H_2O_2 in the cerebral circulation (42). Thus, support exists for a physiological role of superoxide generation through eNOS “uncoupling”.

There are numerous antioxidants that regulate free radicals and ROS including vitamin E, vitamin C, and carotenoids. However, SOD appears to be the primary regulator of ROS in vascular cells. GPx and catalase also function as an important anti-oxidant in vascular cells. There are five isoforms of GPx, and all of them catalyze the reduction of H_2O_2 to H_2O using glutathione as the electron donor (9). Similar to GPx, catalase catalyzes the breakdown of H_2O_2 ; however, GPx has a higher affinity for H_2O_2 at low concentrations ($K_m = 1 \mu M$) than catalase (K_m

= 1 mM), indicating that GPx is a less effective antioxidant at higher concentrations of H₂O₂ (90). Together, these antioxidants regulate O₂⁻-derived ROS, primarily H₂O₂.

SOD-1, located in the cytosol and nucleus and to a lesser extent in the mitochondria, is the predominant isoform of SOD in the vasculature (105, 106). The effects of age on SOD-1 expression and activity are inconclusive. For example, SOD-1 is reduced with age in skeletal muscle arterioles (145); however, SOD-1 activity increases with age in mouse aorta (58). In coronary arterioles, SOD-1 is unaffected by age (28). Exercise training increases SOD-1 protein content in coronary arterioles, aortic endothelial cells, and mouse aorta (50, 113, 114). Elevations in shear stress induce SOD-1 mRNA and protein content in skeletal muscle arteries (66, 146). Although, SOD-1 content declines with age in resistance arteries from skeletal muscle (145) and mesentery (127), the interactive effects of age and exercise training on the expression of antioxidant proteins have not been thoroughly investigated in the skeletal muscle resistance vasculature.

Tempol, which is an SOD mimetic, is widely used to assess the effect of O₂⁻ on vascular reactivity. Many studies report a beneficial effect of scavenging excess O₂⁻ with Tempol on endothelial function; however, most of these studies have been conducted in diseased animals (3, 46, 95, 153, 154). In contrast, SOD treatment diminished acetylcholine-induced vasodilation in cerebral arteries from healthy mice (42). Furthermore, Tempol had no effect on endothelium-dependent vasodilation in cerebral arteries from young rats, but improved endothelium-dependent vasodilation in arterioles from old rats (86). The differences in these reports are probably due to the complexity of pathologies, vasculature beds, and species examined; however, these discrepant results of SOD treatment indicate that the effects of O₂⁻ on endothelial function remain unresolved.

It is well established that exercise training delays and/or reverses the development of many pathologies including cardiovascular disease and endothelial dysfunction. Ironically, a paradox

emerges as these exercise-induced improvements in overall health observed are accompanied by increases in ROS generation. However, the mechanisms by which exercise training improves endothelium-dependent vasodilation remain unknown. It is plausible that the improvement in endothelial function observed with exercise training is linked to elevations of ROS that accompany exercise. Low to moderate O_2^- and ROS are required as modulators of normal cell function and serve as critical signaling molecules in the vasculature (20, 73). eNOS “uncoupling” may contribute to normal vascular function. The interactive effects of age and exercise training on regulation of vascular ROS remain unclear. Furthermore, the role of ROS in mediating age- and exercise training-induced adaptations of endothelial function has not been determined. Therefore, the overall aims of this dissertation were as follows:

- **To determine the effects of O_2^- and ROS on endothelium-dependent vasodilation in skeletal muscle arterioles from young and old, sedentary and exercise trained rats.**
- **To directly measure flow-induced production of NO and O_2^- in skeletal muscle arterioles from young and old, sedentary and exercise trained rats.**
- **To determine the effects of age and exercise training on protein content of enzymes involved in regulation of ROS in skeletal muscle arterioles from young and old, sedentary and exercise trained rats.**

Chapter II

Effect of aging and exercise training on eNOS uncoupling in skeletal muscle arterioles

Overview

Reduced availability of tetrahydrobiopterin (BH₄) contributes to the age-related decline of nitric oxide (NO)-mediated dilation in soleus muscle arterioles. Depending on the availability of substrate and/or necessary co-factors, endothelial nitric oxide synthase (eNOS) can generate NO and/or superoxide (O₂⁻). We evaluated the effects of age and exercise on flow-induced vasodilation and production of NO and O₂⁻ in soleus muscle arterioles. Young (3 mo) and old (22 mo) male rats were exercise trained (ET) or remained sedentary (SED) for 10 wks. Flow-induced NO and O₂⁻ production, as well as BH₄ and L-arginine content, were determined in soleus muscle arterioles. Flow-induced vasodilation was assessed under control conditions and during the blockade of O₂⁻ and/or hydrogen peroxide. Exercise training enhanced flow-induced vasodilation in arterioles from young and old rats. Old age reduced BH₄ levels and flow-induced NO production in SED rats; however, exercise training restored BH₄ and flow-induced production of NO. Flow-induced eNOS-dependent O₂⁻ production was higher in arterioles from old SED compared to those from young SED rats. Exercise training increased flow-induced eNOS-dependent O₂⁻ production in arterioles from young but not old rats. O₂⁻ scavenging with Tempol reduced flow-induced vasodilation in arterioles from all groups except young SED rats. The addition of catalase to Tempol-treated arterioles eliminated flow-induced vasodilation in arterioles from all groups of rats. Catalase alone reduced flow-induced vasodilation in arterioles from all groups except old SED rats. In Tempol-treated arterioles, flow-induced vasodilation was restored by deferoxamine, an iron chelator. These data indicate that uncoupling of eNOS contributes to the age-related decline in flow-induced vasodilation; however, reactive oxygen species are required for flow-induced vasodilation in soleus muscle arterioles from young and old rats.

Introduction

Maximal exercise capacity declines with advancing age (47, 104) and, although part of this decline is associated with a reduction of maximal cardiac output (76), alterations in the local control of skeletal muscle blood flow also contribute to this abatement. Local regulatory factors that contribute to the age-associated decrement in skeletal muscle blood flow capacity include declines in vascular conductance (39). Numerous studies have demonstrated that age impairs endothelial function in skeletal muscle conduit and resistance arteries in humans and animals (38, 54, 97, 145). Age-associated reductions in endothelium-dependent dilation of skeletal muscle resistance arterioles occur in part, through reduced nitric oxide (NO) signaling (122). Mechanisms that may underlie this age-associated impairment in NO signaling may include reduced availability of substrate (L-arginine) (93) or cofactors (e.g., tetrahydrobiopterin [BH₄]) (23, 135), reduced endothelial NO synthase (eNOS) protein levels and/or activity, and increased superoxide production (29). Depending on the availability of L-arginine and/or BH₄, eNOS can become uncoupled resulting in the generation of NO and superoxide (O₂⁻) (24, 137). Aging-induced diminution of BH₄ contributes to the decline of NO-mediated dilation to flow, and sepiapterin, a precursor to BH₄ synthesis (6), improves flow-induced vasodilation in soleus muscle arterioles of aged rats (33). BH₄ supplementation ameliorates endothelium-dependent dilation in humans with cardiovascular diseases or elevated risk factors (80, 126); however, the impact of reduced BH₄ 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, O₂⁻ rapidly interacts with NO to form peroxynitrite, (ONOO⁻) a potent ROS, which elicits cellular damage (14, 29). Secondly, the presence of ROS, and primarily ONOO⁻, reduces BH₄ to the inactive trihydrobiopterin radical (BH₃), thereby reducing this necessary co-

factor and subsequent NO production. Recent reports indicate that insufficient BH₄ is the major determinant of whether eNOS produces O₂⁻ or NO (8, 26).

Aerobic exercise-training restores age-associated reductions in NO-mediated dilation in human and animal models (38, 122, 130). Exercise training may increase NO bioavailability, in part, through enhanced regulation of ROS (113) or through increased expression of eNOS protein (122); however, the effects of age and exercise training on the regulation of eNOS activity have not been evaluated. Eskurza et al., demonstrated that an acute bolus of BH₄ augments endothelium function in the brachial artery of old sedentary men, but had no effect on endothelial-dependent dilation in habitually active, age-matched counterparts (45). These data suggest that aerobic exercise training prevents the loss of NO bioavailability by preserving BH₄ in aged individuals; however, the effect of exercise training on BH₄ levels in the resistance vasculature of skeletal muscle has not been determined. Here, we investigate the effects of age and exercise training on BH₄ availability and eNOS uncoupling in soleus muscle arterioles of male rats.

Therefore, the purpose of this study was 3-fold. First, we sought to determine whether age-induced reductions of arteriolar BH₄ are associated with eNOS uncoupling. Second, we investigated the possibility that exercise training restores arteriolar BH₄ availability and reverses eNOS uncoupling with age. 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 mo) and old (22 mo) male Fischer 344 rats were obtained from Harlan (Indianapolis, IN), 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 (76).

Exercise training

All rats were habituated to treadmill exercise, during which each rat walked on a motor-driven treadmill at 5 m/min (0° incline), 5 min/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 (15° incline), 5 days/wk, for 10–12 wk. The duration of running was gradually increased in the first 3 wk until a 60-min duration was reached. The rats continued to run 5 days/wk for 60 min/day for the remainder of the 10- to 12-wk training period. Vascular responses were determined at least 24 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 (34, 124).

Microvessel preparation

Rats were anesthetized with isoflurane (5%/O₂ balance) and euthanized by decapitation. The gastrocnemius-plantaris-soleus muscle group was dissected free from both hind limbs and placed in a cold (4°C), filtered physiological saline solution (PSS) containing 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl₂, 1.17 mM MgSO₄, 1.2 mM NaH₂PO₄, 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, composed primarily of high-oxidative fibers (34), as previously described.

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 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 (Power Lab). 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 cmH₂O, 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. 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 cm H₂O. Volumetric flow (Q) was then calculated from inner diameter (d) and mean red cell velocity (V_{rbc}), according the following equation (32, 74, 97):

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

At the end of the experiment, arterioles were placed in Ca²⁺-free PSS with 100 μM of the NO donor, sodium nitroprusside for 1 hr to obtain the maximal passive diameter (97, 122).

Effects of ROS Scavengers on Flow-Induced Vasodilation

To determine the role of O_2^- , hydrogen peroxide (H_2O_2), and hydroxyl ion (OH^-) in age-induced reductions of flow-induced vasodilation, responses to flow were evaluated in the presence of the following: (1) superoxide dismutase mimetic, Tempol (100 μ M) (21), (2) H_2O_2 scavenger, catalase (100U) (44), (3) Tempol (100 μ M) plus catalase (100U), (5) NADPH oxidase inhibitor, apocynin (100 μ M) (153), and (6) Tempol plus deferoxamine (100 μ M) (131), an iron chelator, and inhibitor of OH^- formation (131).

Detection of Flow-Induced NO Production

To evaluate flow-induced production 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) (71). The intensity of the fluorescent signal is proportional to NO levels. Following intraluminal loading of DAF-2DA (2.5 μ M) for 20 minutes, soleus muscle arterioles were exposed to a flow rate of 34 nl/sec and fluorescent images were acquired every 15 seconds over 2 minutes. To confirm that production 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 μ M Dea-NONOate (DEA), an NO donor, was used as a positive control to ensure that the dye was not saturated and to determine maximal fluorescent intensity as a control for loading of dye. After background subtraction, DAF fluorescence was expressed as a ratio of DAF stimulated/DAF at baseline for all conditions. Acquired images were analyzed using ImageJ software (NIH).

Detection of Flow-Induced O_2^- Production

Dihydroethidium (DHE) fluorescence was used to evaluate flow-induced O_2^- production in real time (128). To evaluate O_2^- production 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. (128). Following intraluminal loading of DHE (40 μ M) for 20 minutes, soleus muscle arterioles were exposed to a flow rate of 34 nl/sec and fluorescent images were acquired every 15 seconds over 2 minutes. To determine whether production of O₂⁻ occurred through eNOS, flow-induced 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. After background subtraction, DHE fluorescence was expressed as a ratio of DHE stimulated/DHE at baseline for all conditions. Acquired images were analyzed using ImageJ software (NIH).

Arteriolar L-arginine and BH₄ measurements

Arteriolar L-arginine and BH₄ levels were determined using the HPLC method as previously described (33, 89, 148). Briefly, soleus muscle arterioles were dissected from each animal and pooled. For L-arginine analysis, vessels were homogenized with 0.2 ml 1.5 M HClO₄, then 0.1 ml 2 M K₂CO₃ 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 (148). For BH₄ analysis, arterioles were homogenized in 0.1 ml 0.1 M phosphoric acid containing 5 mM dithioerythritol (an antioxidant), to which 17.5 μ l 2 M trichloroacetic acid was added. Extracts were oxidized with acidic or basic iodine. Acidic oxidation quantitatively converts BH₄ and dihydrobiopterin to biopterin; basic oxidation converts dihydrobiopterin and BH₄ 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 analyzed on a C18 reversed-phase column using fluorescence detection and authentic biopterin as a standard. The amount of BH₄ in the arteriolar extracts was determined from the difference between acidic and basic iodine-generated biopterin (89). The sensitivity of L-arginine and BH₄ analyses by HPLC, which was assessed using detection limits defined as a signal-to-noise ratio of 3, was 5 and 2 μ M,

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 x 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 x 100%). The precision and accuracy for the L-arginine analysis were 1.4% and 1.6%, respectively, and for the BH₄ analysis were 2.0% and 2.3%, respectively. . The values in fmol • (mg tissue)⁻¹ and pmol • (mg tissue)⁻¹ for BH₄ and arginine concentrations, respectively, were calculated on the basis of tissue weight.

Data Analysis

Data are expressed as means ± standard error. 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 cm H₂O 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 percent of maximal vasodilation as previously described (97). 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 BH₄ 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 mean ± SEM. 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. Exercise training reduced body mass in both young and old rats (Table 1.1). Soleus muscle mass increased with age but was unaltered by exercise training (Table 1.1). In contrast, soleus muscle mass to body mass ratio decreased with age and increased with exercise training (Table 1.1). 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 (122).

Vessel characteristics

Maximal intraluminal diameter in soleus muscle arterioles was not different between groups (Table 1.1). The levels of spontaneous tone were similar between groups (Table 1.1). Treatment with Tempol, Tempol plus catalase, catalase alone, apocynin and Tempol plus deferoxamine had no effect on spontaneous tone in any group (Table 1.1).

Vasodilator responses to flow

Vasodilation to intraluminal flow was diminished in soleus muscle arterioles from old SED rats (Fig. 1.1). Exercise training restored flow-induced vasodilation in soleus muscle arterioles from old rats to that of young SED rats and exercise training improved flow-induced dilation in soleus muscle arterioles from young rats (Fig. 1.1).

Arteriolar BH₄ and L-arginine content

Similar to previous findings, age decreased arteriolar BH₄ levels (33). Exercise training restored arteriolar BH₄ levels of old rats to that of young rats (Fig. 1.2A). Exercise training did not alter BH₄ levels in soleus muscle arterioles from young rats. As previously reported (33), aging had no effect on L-arginine levels and exercise training did not effect arteriolar L-arginine levels in either young or old rats (Fig. 1.2B).

Flow-induced NO production in soleus muscle arterioles.

Age reduced flow-induced NO production in soleus muscle arterioles (Fig. 1.3). Exercise training augmented flow-induced NO production in soleus muscle arterioles of old rats to that of young SED rats. Exercise training increased flow-induced NO production in soleus muscle arterioles of young rats. L-NAME eliminated flow-induced increases in NO in arterioles from all groups of rats, confirming DAF specificity (Fig. 1.3). Maximal DAF fluorescence was not different between 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) confirming that similar dye loading in arterioles from all groups.

Flow-induced O₂⁻ production in soleus muscle arterioles

Flow-induced O₂⁻ generation was elevated in soleus muscle arterioles from old SED rats as compared to those from young SED rats (Fig 1.4). Exercise training increased flow-induced O₂⁻ production in arterioles of young rats, but did not alter flow-induced O₂⁻ production in old rats, indicating that O₂⁻ signaling contributes to exercise training-induced improvement of flow-induced vasodilation in young rats. Inhibition of eNOS activity with L-NAME reduced flow-induced O₂⁻ production in arterioles from all but young SED rats (Fig. 1.4). L-NAME inhibition of eNOS-derived O₂⁻ was greater in arterioles from old SED rats as compared to those from young SED rats. Exercise training increased eNOS-derived O₂⁻ production in soleus muscle arterioles from young

but not old rats (Fig. 1.4). Maximal DHE fluorescence 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 similar dye loading in arterioles from all groups.

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

In young SED rats, scavenging of O_2^- with Tempol did not alter flow-induced vasodilation. Catalase, and Tempol plus catalase inhibited flow-induced vasodilation indicating dependence on H_2O_2 in young SED rats (Fig 1.5A).

Scavenging of O_2^- with Tempol inhibited flow-induced vasodilation in arterioles from old SED rats (Fig. 1.5B). Scavenging of H_2O_2 with catalase reduced flow-induced vasodilation between flow rates of 5.2 ± 0.75 to 22.8 ± 0.42 nl/sec but not at higher flow rates, and Tempol plus catalase eliminated flow-induced vasodilation in the old SED rats, suggesting that even though flow-induced vasodilation is reduced in these rats compared to young SED, H_2O_2 is an important vasodilator (Fig. 1.5B).

In young and old ET rats, scavenging of O_2^- with Tempol reduced flow-induced vasodilation (Fig. 1.5C and D). Scavenging of H_2O_2 with catalase alone reduced flow-induced dilation in arterioles from young ET rat (Fig. 1.5C). Similarly, simultaneous scavenging of both O_2^- and H_2O_2 with Tempol plus catalase inhibited flow-induced vasodilation in arterioles from young ET rats (Fig. 1.5C); however, some flow-induced vasodilation remained, suggesting that in young ET rats, flow-induced vasodilation depends on both H_2O_2 and NO. In old ET rats, H_2O_2 scavenging with catalase alone reduced flow-induced vasodilation (Fig. 1.5D) and scavenging of O_2^- and H_2O_2 with Tempol plus catalase abolished flow-induced vasodilation (Fig. 1.5D) indicating that H_2O_2 is critical for flow-induced vasodilation in old ET rats.

Next, we determined the contribution of NADPH oxidase-derived O_2^- to flow-induced vasodilation in young and old, SED and ET rats. The inhibition of NADPH oxidase with

apocynin significantly reduced flow-induced vasodilation in young and old SED rats (Fig 1.6A); however, apocynin had no effect on flow-induced vasodilation in young and old ET rats (Fig. 1.6B) suggesting that O_2^- -derived H_2O_2 required for flow-induced vasodilation in the ET rats is not generated from NADPH oxidase-derived O_2^- .

Role of OH⁻ in flow-induced vasodilation

SOD has the potential to generate OH^- in the presence of iron and OH^- may cause vasoconstriction (12). The addition of deferoxamine, an iron chelator, to Tempol-treated arterioles had no effect in young SED rats (Fig 1.7A); however, iron chelation restored the Tempol-induced loss of flow-induced vasodilation in arterioles from old SED (Fig 1.7B) indicating that OH^- generated is eliciting a vasoconstriction. Deferoxamine added to Tempol-treated arterioles improved the Tempol-induced loss of flow-induced vasodilation observed in young and old ET rats (Fig. 1.7C 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.

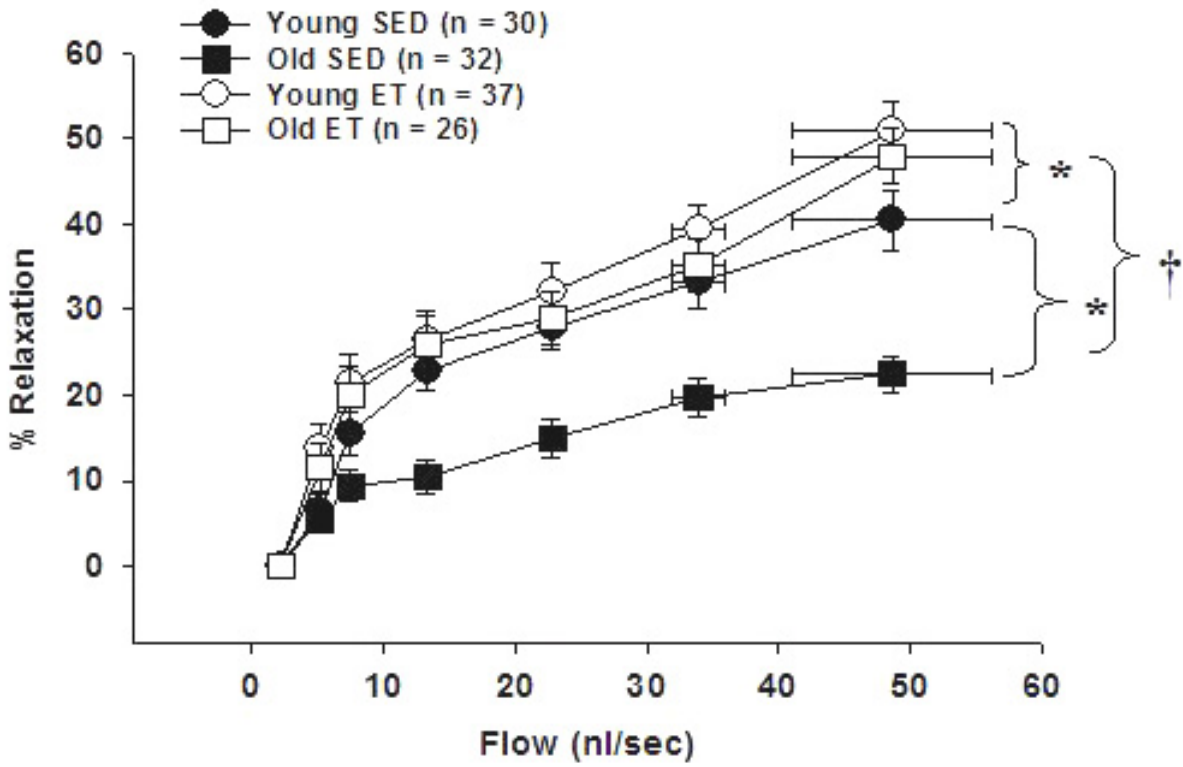


Figure 1.1 Diameter changes in response to increasing intraluminal flow in soleus muscle arterioles. Exercise training restores flow-induced vasodilation in old and improves flow-induced vasodilation in young rats. * $P < 0.05$ vs. YSED and † $P < 0.05$ vs. OSED.

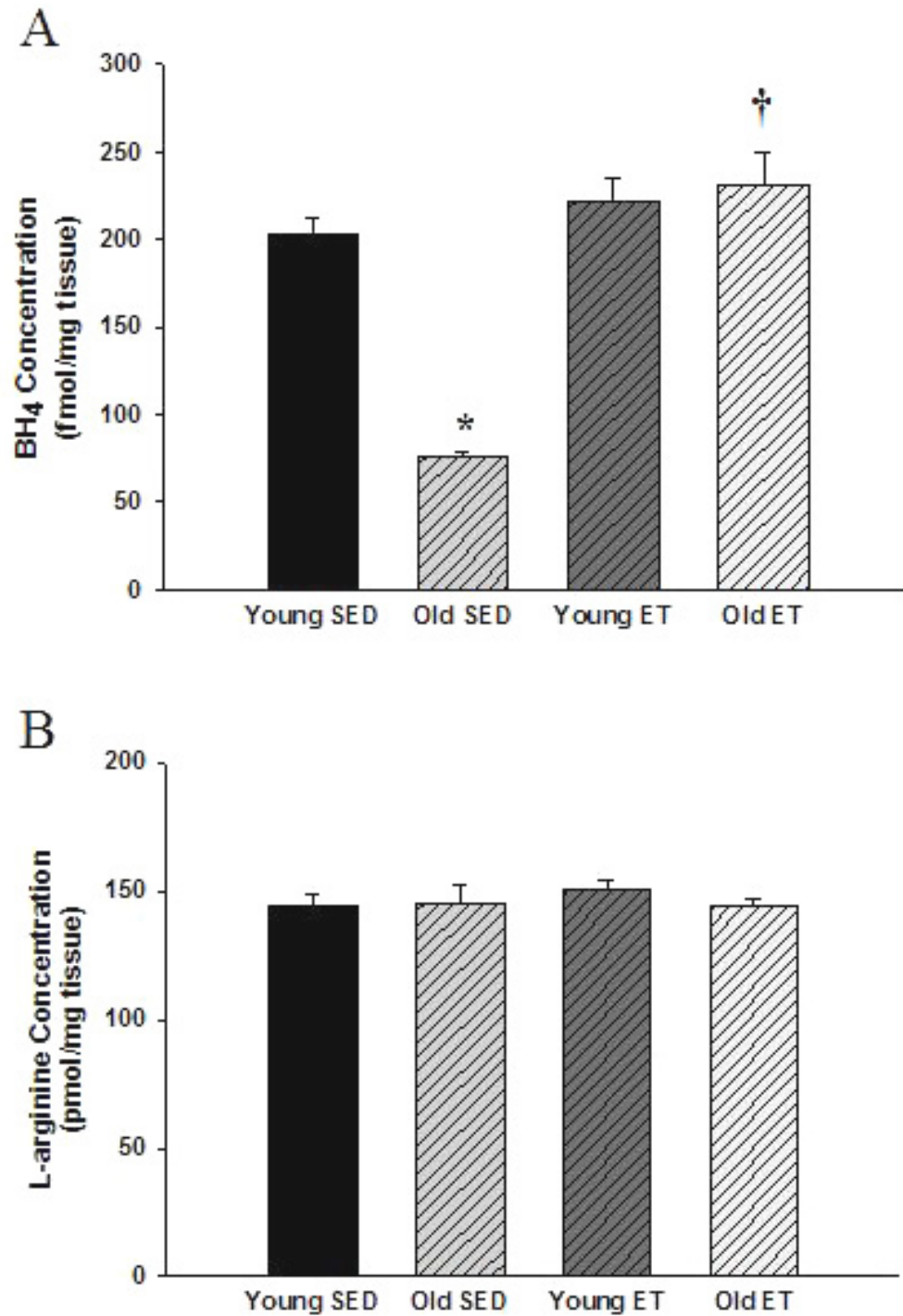


Figure 1.2. Arteriolar BH₄ and L-arginine content in soleus muscle arterioles from young and old, sedentary and exercise-trained rats. (A) Exercise training restores age-related declines in arteriolar BH₄ levels in soleus muscle arterioles. (B) Aging and exercise training had no effect on arteriolar L-arginine levels in soleus muscle arterioles. **P* < 0.05 vs. YSED and † *P* < 0.05 vs. OSED. n = 6-8 per group.

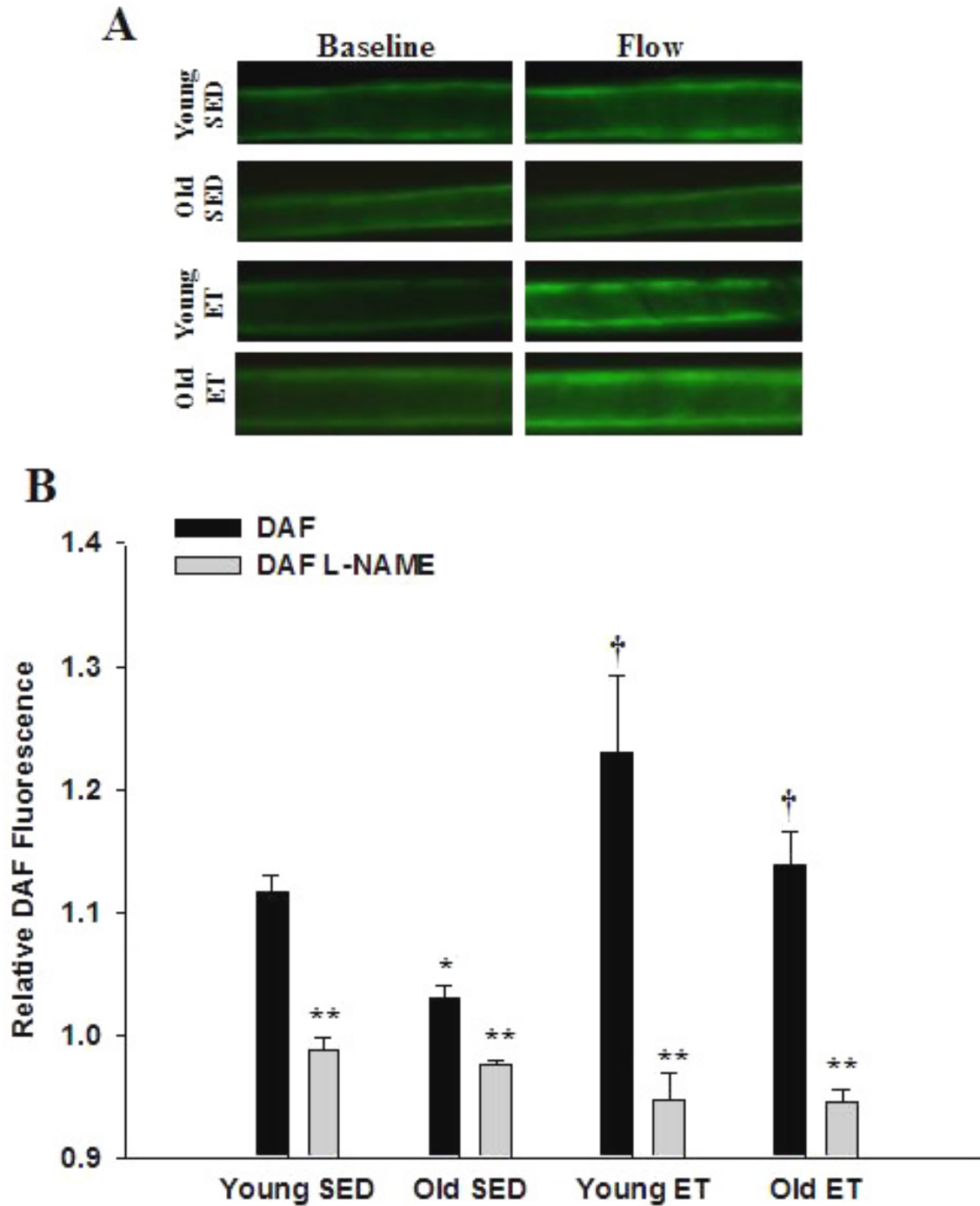


Figure 1.3. Flow-stimulated DAF fluorescence in soleus muscle arterioles from young and old, sedentary and exercise-trained rats. (A) DAF fluorescence indicates NO bioavailability in soleus muscle arterioles from young and old, sedentary and exercise trained rats at baseline and during flow stimulation (34 nl/s). (B) Summarized data indicate that flow-stimulated DAF 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 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.

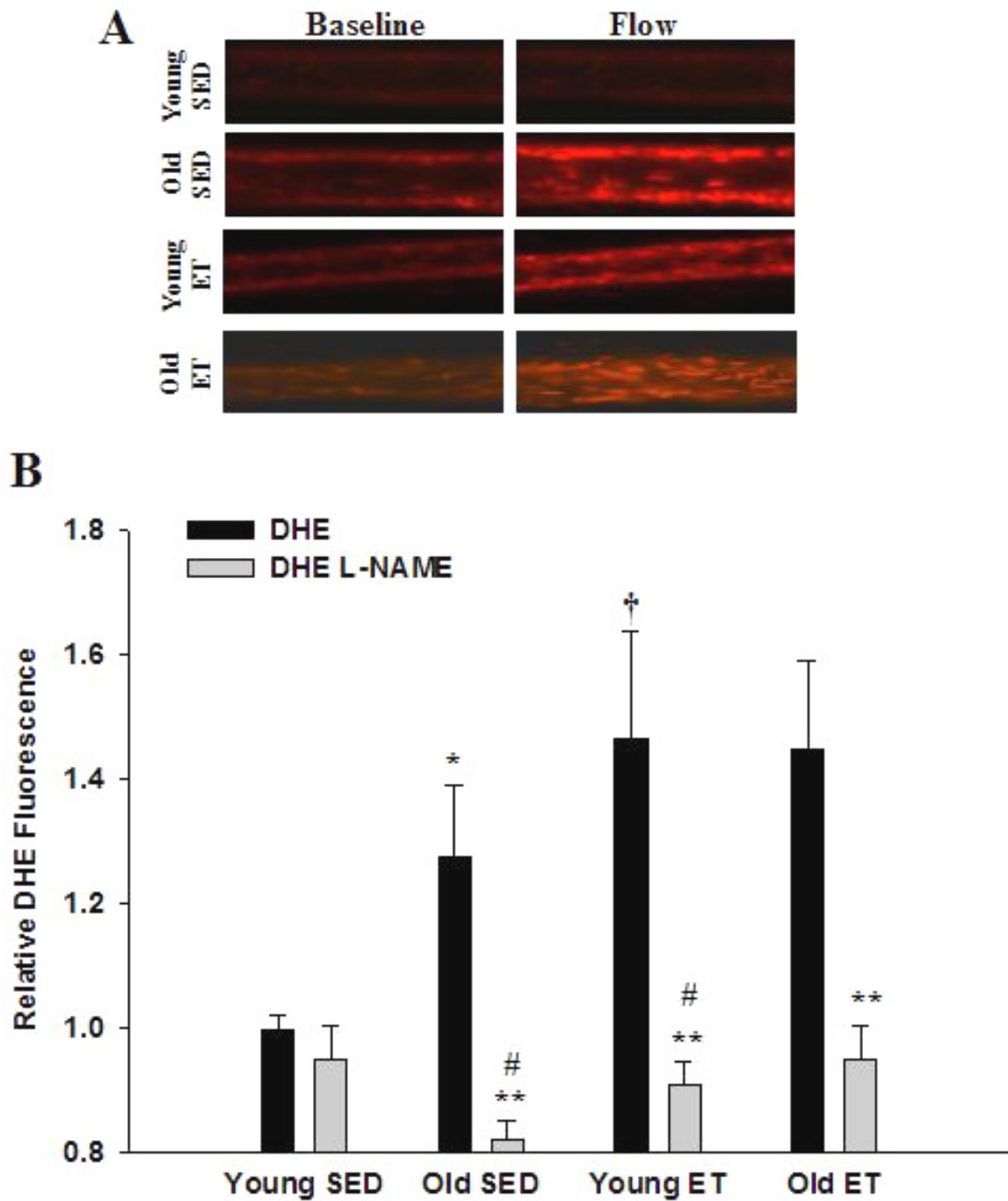


Figure 1.4. Flow-induced DHE fluorescence (an indicator of superoxide production) in soleus muscle arterioles of young and old, sedentary and exercise-trained rats. (A) DHE fluorescence indicates O_2^- production in soleus muscle arterioles from young and old, sedentary and exercise trained rats at baseline and during flow stimulation (34nl/s). (B) Summarized data indicate that flow stimulated an increase in DHE fluorescence in soleus muscle arterioles from old SED rats. Exercise training increased DHE fluorescence in young rats; however, had no effect in old rats. L-NAME blocks DHE fluorescence 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 # $P < 0.05$ Δ in DHE fluorescence with L-NAME vs. young SED. $n = 9 - 11$ per group.

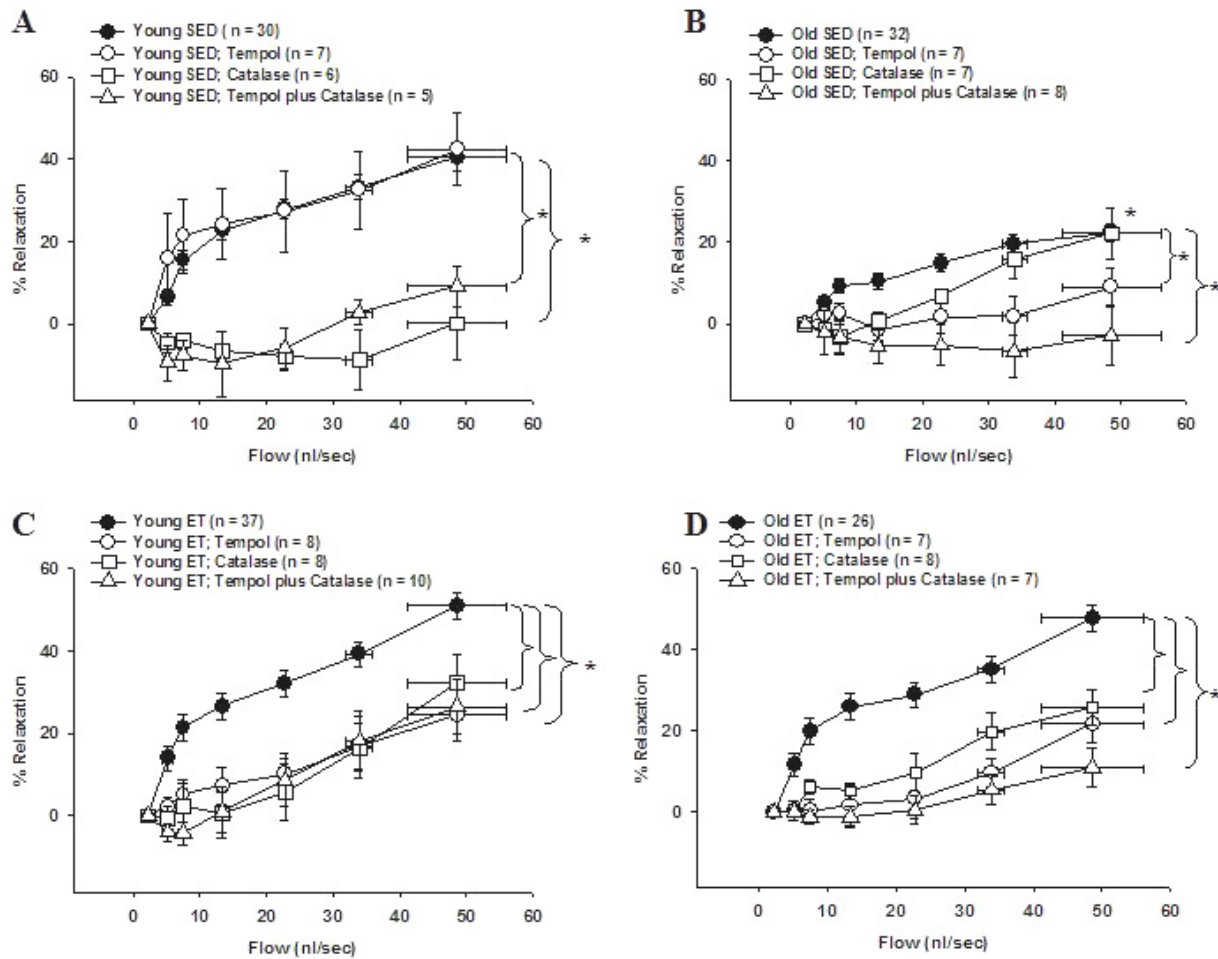


Figure 1.5. Effects of Tempol and catalase on flow-induced vasodilation of soleus muscle arterioles from young and old, sedentary and exercise-trained rats. (A) In young SED rats, Tempol had no effect, but catalase alone and Tempol + catalase completely abolished flow induced vasodilation, (B) In old SED rats, catalase had no effect but Tempol and Tempol + catalase reduced flow induced vasodilation, (C) All treatments reduced flow-induced vasodilation in young ET, and (D) old ET rats. * $P < 0.05$ vs. control response.

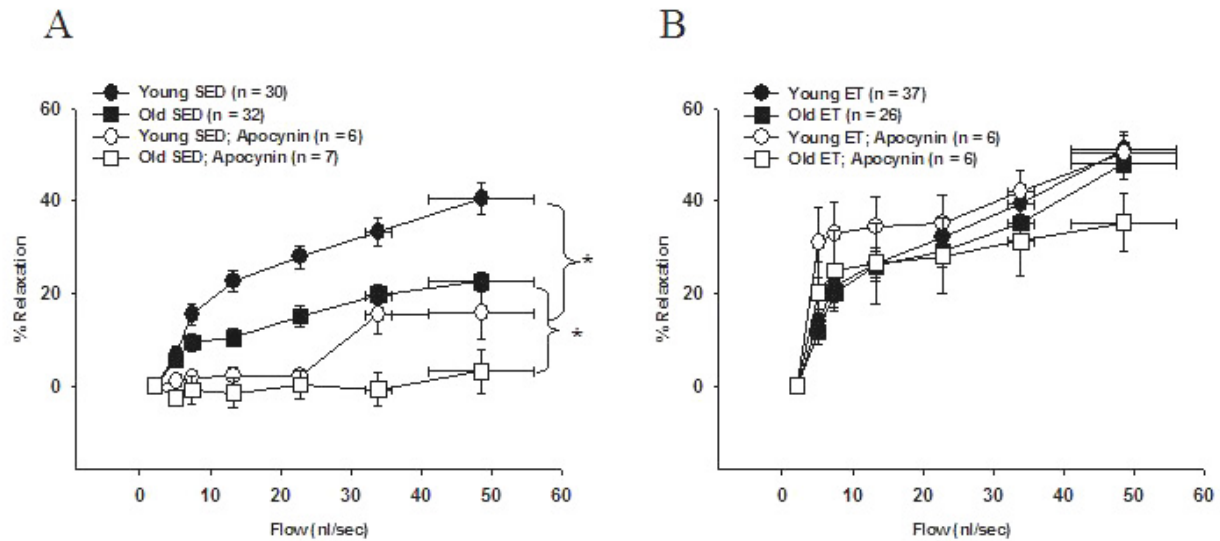


Figure 1.6. Role of NADPH oxidase-derived O_2^- production in flow-induced vasodilation in soleus muscle arterioles of young and old, sedentary and exercise-trained rats. (A) NADPH oxidase inhibition with Apocynin reduced flow-induced vasodilation in soleus muscle arterioles of young and old SED rats, (B) Inhibition of NADPH oxidase with apocynin had no effect on flow-induced vasodilation in soleus muscle arterioles of young and old ET rats. * $P < 0.05$ vs. control response.

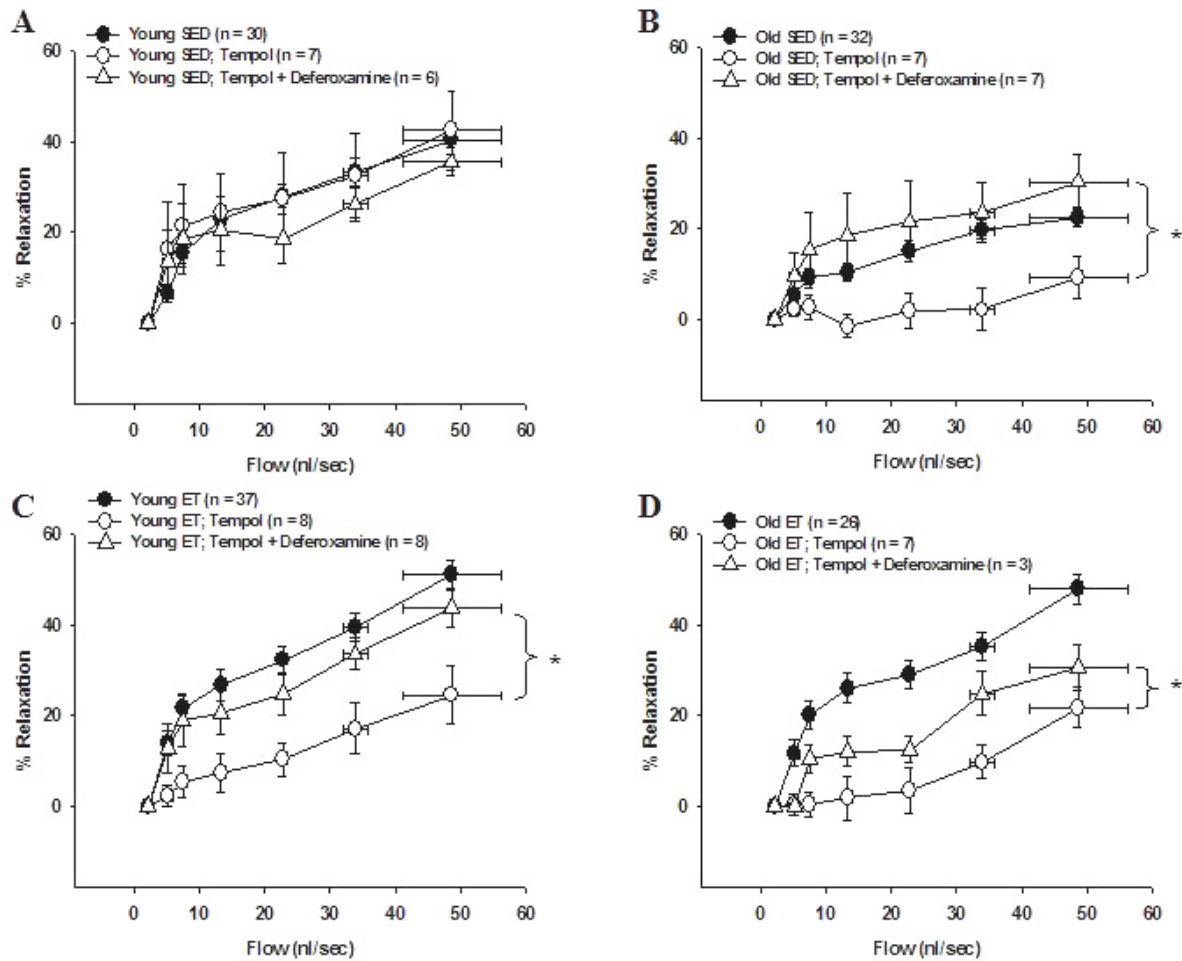


Figure 1.7. Effects of Tempol and Deferoxamine on flow-induced vasodilation in soleus muscle arterioles of young and old, sedentary and exercise-trained rats. (A) In young SED rats, Tempol and Tempol + Deferoxamine, an iron chelator, had no effect on flow-induced vasodilation, (B) In old SED rats, Tempol reduced and Tempol + Deferoxamine improved flow-induced vasodilation, (C) In young ET rats, Tempol reduced and Tempol + Deferoxamine restored flow-induced vasodilation, (D) In old SED rats, Tempol reduced flow-induced vasodilation; however, Tempol + Deferoxamine improved flow-induced vasodilation in the old ET. * $P < 0.05$ vs. control response.

	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 ± 6 *	379 ± 5 †
Soleus muscle wt (mg)	155.9 ± 3.8	164.6 ± 3.0	165.7 ± 3.2*	168.8 ± 3.9
Soleus wt/Body wt (mg/g)	.41 ± 0.01	.47 ± 0.01*	.37 ± 0.01*	.45 ± 0.01†
Vessel Characteristics				
Maximal Diameter (µm)	130 ± 4	127 ± 4	123 ± 4	124 ± 5
Spontaneous tone (%)	51 ± 2 (n=30)	54 ± 2 (n=37)	51 ± 2 (n=33)	57 ± 2 (n=26)
with Tempol	48 ± 5 (n=7)	47 ± 5 (n=8)	61 ± 6 (n=7)	59 ± 3 (n=10)
with Tempol + Catalase	50 ± 5 (n=5)	53 ± 4 (n=10)	54 ± 3 (n=10)	57 ± 3 (n=7)
with Catalase	45 ± 2 (n=6)	53 ± 5 (n=9)	55 ± 3 (n=7)	56 ± 6 (n=9)
with Apocynin	46 ± 3 (n=6)	52 ± 5 (n=6)	57 ± 4 (n=7)	56 ± 4 (n=6)
with Tempol + Deferoxamine	54 ± 4 (n=6)	48 ± 5 (n=9)	64 ± 3 (n=7)	60 ± 5 (n=3)

Table 1.1. Soleus muscle and vessel characteristics of young and old, sedentary and exercise-trained rats. Maximal diameter was recorded in Ca²⁺-free physiological saline solution with 100µM sodium nitroprusside. Tone (%) = [(maximal diameter – diameter with tone)/maximal diameter] × 100. * *P* < 0.05 vs. YSED and † *P* < 0.05 vs. old SED.

Discussion

Several important new findings emerge from this study. (1) The balance between O_2^- and NO signaling is altered by age and exercise training in skeletal muscle arterioles. (2) BH_4 content is reduced with age and restored with exercise training in skeletal muscle arterioles from old rats. The age-related decline in arteriolar BH_4 content is concomitant to a decrease in NO signaling and an increase in ROS signaling. The exercise-induced restoration of BH_4 content potentially contributes to increased NO signaling in skeletal muscle arterioles of old ET rats. (3) In conditions of limited BH_4 , such as occurs with age, NO-mediated signaling declines and eNOS-generated ROS (O_2^-) increases in skeletal muscle arterioles. (4) Exercise training increases both NO- and ROS-mediated signaling in skeletal muscle arterioles of young rats, but a balance in these signaling systems is maintained, resulting in robust flow-induced vasodilation.

Aging is associated with declines in flow-induced NO-dependent vasodilation in conduit and resistance arterioles of humans and rats (38, 54, 97, 145). 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 vasodilation in skeletal muscle arterioles (33). When BH_4 is limited, eNOS becomes uncoupled, producing both O_2^- and NO (8, 26). Eskurza et al. reported that an acute bolus of BH_4 restored flow-mediated dilation in older sedentary humans (45) and the BH_4 precursor, sepiapterin, augments flow-induced vasodilation in skeletal muscle arterioles in aged rats (33). These findings support the hypothesis that the age-associated decrements in BH_4 contribute to reduced NO bioavailability. We tested the hypothesis that eNOS uncoupling increases with age in skeletal muscle arterioles due to lack of BH_4 . To our knowledge, this is the first study to demonstrate that age-related reduction of BH_4 (Fig. 1.2A) is accompanied

by impaired flow-induced NO signaling (Fig. 1.3) and increased eNOS-derived O_2^- production in skeletal muscle arterioles.

Increases in O_2^- production, observed with age, scavenge NO and may alter endothelial function (40). In soleus muscle feed arteries, SOD-1 protein content, but not mRNA expression, is reduced in aged rats (145); however, SOD-1 activity appears to increase in the aorta of aged rats (37). Additionally, catalase activity declines in aorta from aged rats (37); however, catalase protein content does not change with age in human endothelial cells (40). Exercise training potentially improves NO bioavailability by enhanced regulation of O_2^- and ROS by inducing antioxidant defense systems and reducing O_2^- generating systems. Rush *et al.* 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 (113). Graham and Rush further demonstrated that the cytosolic subunit of NADPH oxidase, gp91phox, is reduced with training in aorta homogenates from young pigs (56). Based on these observations, it appears that exercise training improves the management of superoxide (and subsequent ROS) generation by enhancing antioxidant mechanisms and reducing pro-oxidant pathways. However, a paradox emerges as O_2^- generation and oxidant stress increases with exercise training (4, 5, 30). Our data suggest that exercise training-induced enhancement of flow-induced vasodilation occurs because both NO and ROS mediated signaling increase in a balanced fashion.

Even though high levels of ROS may potentiate the pathology of cardiovascular diseases (62), it is becoming increasingly clear that ROS can serve as signaling molecules, which mediate normal cellular functions (41). H_2O_2 generated by the dismutation of O_2^- is emerging as an important vasodilator and signaling molecule in the vasculature. Endothelium-derived H_2O_2 contributes to flow-induced vasodilation in isolated coronary arterioles (72). Marvar *et al.* demonstrated that H_2O_2 produced by contracting skeletal muscle elicits a vasodilatory response on

nearby skeletal muscle arterioles (82). Furthermore, exogenous H₂O₂ is vasodilatory in a variety of vascular beds including pulmonary (13), skeletal muscle (27), cerebral (139) and mesenteric beds (84). H₂O₂ also indirectly alters vascular reactivity by activating eNOS via phosphoinositide 3-kinase (PI3K) (133). Our data indicate that H₂O₂ is an important mediator of flow-induced vasodilation of skeletal muscle arterioles from young SED rats. In addition, our data reveal that flow-induced H₂O₂ signaling declines with age, but is restored by exercise training.

SOD is an important anti-oxidant enzyme that reduces oxidant stress by dismutating O₂⁻ into H₂O₂; however, in the presence of catalytic transition metals, SOD can rapidly form OH⁻ (107). H₂O₂ generates OH⁻ through metal-catalyzed reactions, such as the Fenton reaction as follows: $\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \bullet\text{OH} + \text{OH}^-$. The formation of OH⁻ is further promoted by the presence of O₂⁻, which reacts with Fe³⁺ to produce Fe²⁺ through the Haber-Weiss reaction (60, 108). The net effect of SOD is the dismutation of O₂⁻ to produce either the vasodilatory H₂O₂ or in the presence of Fe²⁺, OH⁻, which is a potent vasoconstrictor. Iron chelators, such as deferoxamine, inhibit the generation of OH⁻ by preventing iron ions from catalyzing redox reactions, thereby improving vasodilation in coronary arterioles (101, 131). Iron accumulation increases with age in skeletal muscle, liver, and cardiac muscle, and is associated with increased oxidative stress and diminished functional capacity (12, 150). Age-associated increases in iron accompanied by diminished catalase activity (37) can produce excess OH⁻ contributing to decreased flow-induced vasodilation in skeletal muscle arterioles of old rats (Fig. 1.2B). Exercise training increases iron in skeletal muscle of rats (99) and catalase activity in the vasculature remains unchanged with exercise training (113). In conjunction with increases in O₂⁻ generation with exercise training observed in the present study, elevated OH⁻ production may result when arterioles are treated with an exogenous SOD. Increased OH⁻ production is a potential

mechanism by which iron chelation improves SOD-associated decrements in flow-induced vasodilation in skeletal muscle arterioles.

In conclusion, the results of the present study confirm previous findings that aging impairs endothelium-dependent, NO-mediated vasodilation and BH₄ levels in rat skeletal muscle arterioles (33, 97). The present findings further demonstrate that limited availability of BH₄, as observed in old SED rats, may contribute to eNOS uncoupling, and both a subsequent decrease in NO signaling and an increase in eNOS-derived O₂⁻ generation. Exercise training restores BH₄ levels and improves flow-induced NO production in arterioles from aged rats. Furthermore, exercise training increases both NO and ROS-mediated signaling in skeletal muscle arterioles, suggesting that exercise training-induced enhancement of flow-induced vasodilation in skeletal muscle arterioles involves a balance between NO and O₂⁻-derived ROS. Our results suggest that the beneficial effects of exercise training in the skeletal muscle resistance vascular involve much more than improvement of NO bioavailability.

Chapter III

Age and exercise training alter signaling through reactive oxygen species in the endothelium of skeletal muscle arterioles

Overview

Exercise training ameliorates age-related impairments in endothelium-dependent vasodilation in soleus muscle arterioles. Additionally, exercise training is associated with increased O_2^- production. The purpose of this study was to determine the role of O_2^- and O_2^- -derived reactive oxygen species signaling in mediating endothelium-dependent vasodilation in skeletal muscle resistance arterioles from young and old, sedentary and exercise trained rats. Young (3 mo) and old (22 mo) male rats were either exercise trained (ET) or remained sedentary (SED) for 10 wks. To determine the impact of reactive oxygen species signaling on endothelium-dependent vasodilation, responses to acetylcholine were studied under control conditions and during the scavenging of O_2^- and/or H_2O_2 . Reactivity to O_2^- and H_2O_2 was also determined. Exercise training reversed the age-related impairment of endothelium-dependent vasodilation. Tempol, a scavenger of O_2^- , reduced ACh-induced vasodilation in all groups. Catalase reduced ACh-induced vasodilation in all groups. Similarly, the addition of catalase to Tempol-treated arterioles reduced ACh-induced vasodilation in all groups. Aging had no effect on protein content of SOD-1, catalase, or GPx; however exercise training increased protein content of SOD-1 in young and old rats, catalase in young rats, and GPx in old rats. In summary, exercise training restores endothelium-dependent vasodilation in soleus muscle arterioles. This adaptation is mediated, in part, through enhanced O_2^-/H_2O_2 signaling.

Introduction

Endothelial dysfunction of skeletal muscle declines with age primarily due to decreased nitric oxide (NO) availability (38, 97, 122, 129). In feed arteries of skeletal muscle, reduction of NO-dependent vasodilation is accompanied by reduced expression of endothelial nitric oxide synthase (eNOS) (145). In contrast, we have previously reported that NO-mediated vasodilation of soleus muscle resistance arteries declines with advancing age despite an increase in eNOS protein levels (122). Thus, the age related decline in bioavailability of NO may be dependent upon numerous factors that regulate both its production and degradation. eNOS activity, and subsequent NO production, is regulated by availability of substrate and co-factors, by protein-protein interactions, and by coordinated phosphorylation and dephosphorylation of eNOS (48, 51, 61). Degradation of NO is dependent upon the presence of cellular superoxide (O_2^-), a by-product of cellular respiration, which reacts readily with NO, eliminating its vasodilatory action (121).

Cellular O_2^- is regulated by the enzyme superoxide dismutase (SOD), which catalyzes the dismutation of O_2^- into hydrogen peroxide (H_2O_2). Thus, NO bioavailability and maintenance of NO-mediated vasodilation is linked to SOD activity. In addition, both O_2^- and O_2^- -derived reactive oxygen species (ROS) exhibit vasoactive properties. For example, H_2O_2 has been reported to produce hyperpolarization and relaxation of vascular smooth muscle (84, 91). Furthermore, H_2O_2 regulates eNOS protein expression and activity (133, 152). Thus, tight regulation of O_2^- and O_2^- -derived ROS is necessary for maintenance of optimal endothelium-dependent function, and an age-related increase in vascular O_2^- is a likely contributor to age-induced endothelial dysfunction.

Exercise training reverses age-related reductions in NO bioavailability, thus improving endothelium-dependent vasodilation. In skeletal muscle arterioles of both young and aged rats, exercise training increases eNOS protein expression. This increase in enzyme expression may

contribute to exercise training-induced amelioration of endothelial function in aged rats (122). Additionally, exercise training has been reported to increase vascular expression of SOD. Rush *et al* demonstrated that, in aortic endothelial cells (AEC) from young pigs, SOD-1 protein abundance and activity increased with training (113). Although SOD-1 expression declines with age in resistance arteries from skeletal muscle (145) and mesentery (127), the interactive effects of age and exercise training on the expression of anti-oxidant proteins have not been thoroughly investigated in the skeletal muscle resistance vasculature.

Therefore, the purpose of this study was to determine the role of O_2^- -derived ROS in mediating endothelium-dependent vasodilation in skeletal muscle resistance arterioles from young and old, sedentary and exercise-trained rats. We tested the hypothesis that age-related increases in O_2^- -derived ROS would contribute to endothelium-dependent vasodilation of skeletal muscle arterioles, despite an overall decline in endothelial function. We further hypothesized that exercise training would improve endothelium-dependent dilation, in part, through improved regulation of O_2^- -derived ROS signaling.

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 mo) and old (22 mo) male Fischer 344 rats were obtained from Harlan (Indianapolis, IN), housed under a 12:12-h light-dark cycle, and given food and water *ad libitum*. Fischer 344 rats were chosen because cardiovascular function decreases with age in these rats, without the development of atherosclerosis or hypertension (76).

Exercise training

All rats were habituated to treadmill exercise, during which each rat walked on a motor-driven treadmill at 5 m/min (0° incline), 5 min/day for 3 days. After habituation, young and old rats were randomly assigned to either a control sedentary (SED) group (young SED, n = 37, and old SED, n = 29) or an exercise-trained (ET) group (young ET, n = 25, and old ET, n = 21). ET rats performed treadmill running at 15 m/min (15° incline), 5 days/wk, for 10–12 wk. The duration of running was gradually increased in the first 3 wk until a 60-min duration was reached. The rats continued to run 5 days/wk for 60 min/day for the remainder of the 10- to 12-wk training period. Vascular responses were determined at least 24 h after the last exercise bout in ET rats.

Muscle oxidative enzyme activity

To determine the efficacy of the training protocol, soleus muscle, was stored at –80°C for determination of citrate synthase activity, a measure of muscle oxidative capacity (34).

Microvessel preparation

Rats were anesthetized with isoflurane (5%/O₂ balance) and euthanized 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 CaCl₂, 1.17 mM MgSO₄, 1.2 mM NaH₂PO₄, 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 dissected free of the soleus muscle, composed primarily of high-oxidative fibers (34), as described previously (97). 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 nylon suture. Size and resistance 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 (Power Lab). 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 cmH₂O, 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.

Evaluation of vasodilator responses

Once a steady level of spontaneous tone was achieved, vasodilator responses to the cumulative addition of the endothelium-dependent vasodilator acetylcholine (ACh, 10⁻⁹–10⁻⁴ M) was determined. To evaluate vascular smooth muscle responsiveness to exogenous NO, a concentration-response to diethylamineNONOate (Dea-NONOate, 10⁻⁹–10⁻³ M) was determined. At the end of the concentration–response determinations, arterioles were placed in Ca²⁺-free PSS with 100 μM of the NO donor, sodium nitroprusside, for 1 h to obtain the maximal passive diameter (88, 96, 97).

Evaluation of inhibitory effects of Tempol, Catalase, Tempol + Catalase

To determine the role O₂⁻ and O₂⁻-derived ROS on ACh-induced vasodilatation, responses to ACh were evaluated after a 20-min incubation under one of the following conditions: (1) SOD mimetic Tempol (100 μM) (21), (2) H₂O₂ scavenger, catalase (100U) (42, 44), or (3) Tempol (100 μM) plus catalase (100U). To determine the role of O₂⁻ in scavenging of NO, responses to Dea-NONOate were evaluated after a 20-min incubation with the SOD mimetic, Tempol (100 μM) (21).

Evaluation of vasoreactivity to H₂O₂ and O₂⁻

To determine the reactivity of soleus muscle arterioles to H₂O₂ and a O₂⁻ generator, concentration response curves were generated using authentic H₂O₂ (1e⁻⁶ – 1e⁻² M) and pyrogallol, an O₂⁻ generator (1e⁻⁸ – 1e⁻⁴ M).

Immunoblot analysis of soleus muscle arteriolar protein

Differences in SOD-1, catalase, and glutathione peroxidase-1 (GPx1) protein content in skeletal muscle resistance arterioles were assessed by immunoblot analysis. Arterioles were isolated, snap frozen, and stored at -80°C until analysis. Vessels were lysed in 1X sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 6 M urea, 160 mM DTT, 0.1% bromophenol blue), followed by 3 X 2 minute boil, vortex, and quick spin. After a final 1-minute sonication step, protein concentration was determined using the NanoOrange Protein Quantification Kit (Invitrogen). Samples (10 µg total protein) were subjected to SDS-polyacrylamide gel electrophoresis (10%) and then transferred to nitrocellulose membranes. Membranes were blocked for 1h at room temperature (5% nonfat dry milk in Tris-buffered saline + 0.1% Tween-20) and then incubated overnight at 4° with primary antibodies for SOD-1 1:5000 (Stressgen), catalase 1:6000 (Chemicon), and GPx1 1:4000 (Abcam) or β-actin 1:2000, (Cell Signaling Technologies). After washing, membranes were incubated with respective horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. Peroxidase activity was determined using SuperSignal West Femto (Pierce), with image analysis performed using ImageJ (NIH). Loading differences were normalized by expressing all data as densitometric units for protein of interest relative to β-actin.

Data Analysis

Data are expressed as means \pm standard error (SE). 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 cm H_2O and D_T is the steady-state baseline diameter recorded at the same pressure. The vasodilator responses 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 the respective stage, D_B is the diameter recorded immediately prior to initiation of the concentration-diameter curves, and D_M is the maximal diameter for the arteriole. Concentration-diameter curves were evaluated by a three-way ANOVA with repeated measure on one factor, in order to detect differences within concentrations and between experimental groups. Group differences in animal and vessel characteristics and citrate synthase activity were compared by a two-way ANOVA. Group differences in SOD-1, catalase, or GPx1 protein content were assessed using a two-way ANOVA. In all experiments, n indicated the number of animals studied. Statistical significance was defined as $P \leq 0.05$.

Results

Animals

Body mass increased with age (Table 2.1). Exercise training resulted in lower body mass in both young and old rats. Soleus muscle mass increased with age, but was unchanged by exercise training (Table 2.1). Soleus muscle mass to body mass ratio decreased with age and increased with exercise training (Table 2.1). Exercise training increased citrate synthase activity by approximately 18.3% in soleus muscle of young rats, and by approximately 20.1% in soleus

muscles of old rats (Table 2.1), confirming the efficacy of the exercise training as previously reported (122).

Vessel characteristics

Maximal diameter of soleus muscle arterioles was not different between groups (Table 2.1). The levels of spontaneous tone were similar between groups (Table 2.1) and treatment with Tempol, catalase or Tempol plus catalase, had no effect on spontaneous tone in any group (Table 2.1).

Endothelium-dependent vasodilation to ACh

ACh-induced vasodilation was impaired in soleus muscle arterioles from old SED rats, confirming previous results (122) (Fig. 2.1). Exercise training restored ACh-induced vasodilation in soleus muscle arterioles from old rats to that of young SED rats and improved ACh-induced dilation in young rats at ACh concentrations of $1e^{-5}$ and $1e^{-4}$ M (Fig. 2.1).

Effects of Tempol, catalase and Tempol + catalase on ACh-induced vasodilation

Surprisingly, dismutating O_2^- to H_2O_2 with Tempol, reduced ACh-induced vasodilation in young and old, SED and ET rats (Figs. 2.2A and 2.3A). Furthermore, Tempol eliminated the age-related differences in SED rats suggesting that alterations of O_2^- -signaling contribute to the age-associated impairment in ACh-induced vasodilation. Additionally, SOD in the presence of catalytic transition metals, can rapidly form the vasoconstrictor, hydroxyl radical (OH^\cdot) (107, 131). Iron increases with age and with exercise training in skeletal muscle and may contribute to Tempol-induced OH^\cdot production, most prominently in old ET rats (Fig. 2.3A) (99, 150). Removal of H_2O_2 by catalase reduced ACh-induced vasodilation in all groups of rats (Fig. 2.2B and 2.3B) indicating that H_2O_2 is an important signaling molecule in ACh-induced vasodilation in skeletal muscle arterioles. Simultaneous scavenging of O_2^- and H_2O_2 reduced ACh-induced vasodilation all groups (Fig. 2.2C and 2.3C) and eliminated age differences in SED rats (Fig. 2.2C) revealing

that a proper balance of O_2^- and H_2O_2 must be maintained for ACh-induced vasodilation in SED and ET rats.

Vasoreactivity to H_2O_2 and O_2^- in soleus muscle arterioles

At concentrations above $1e^{-4}$ M, H_2O_2 elicited vasodilation in skeletal muscle arterioles, and these vasodilatory responses were not affected by age or exercise training status (Fig. 2.4). O_2^- generated by the auto-oxidation of pyrogallol (79, 81) did not elicit a vasoactive response in arterioles from either young or old SED rats (Fig. 2.5). In arterioles from young but not old ET rats, pyrogallol elicited a slight vasoconstriction.

Vasodilation to DEA-NONOATE

Confirming previous findings (97), endothelial-independent vasodilation to exogenous NO was preserved with age. In contrast, exercise training increased vasodilation of skeletal muscle arterioles at concentrations of $1e^{-7}$ M Dea-Nonoate in both young and old rats (Fig. 2.6A). Scavenging of O_2^- with Tempol had no effect on endothelium-independent vasodilation in arterioles from any group of rats (Fig. 2.6B and 2.C). Thus, the age-related reduction of NO bioavailability in skeletal muscle arterioles probably does not result from increased scavenging of extracellular NO, demonstrating the specificity of Tempol-induced reductions of vasodilation to ACh (Fig 2.2A and 2.2B).

SOD-1, catalase and GPx1 protein levels

SOD-1 protein levels were not different between arterioles from young and old SED rats. Exercise training increased SOD-1 protein levels by approximately 130% in arterioles from young rats and by approximately 85% in arterioles from old rats (Fig. 2.7A). Catalase levels were not different between arterioles from young and old SED rats. Exercise training increased catalase protein by approximately 81% in arterioles from young rats (Fig. 2.7B). GPx-1 protein levels in

arterioles were unaffected by age; however, exercise training increased GPx-1 protein levels in arterioles from old rats by approximately 66% (Fig 2.7C).

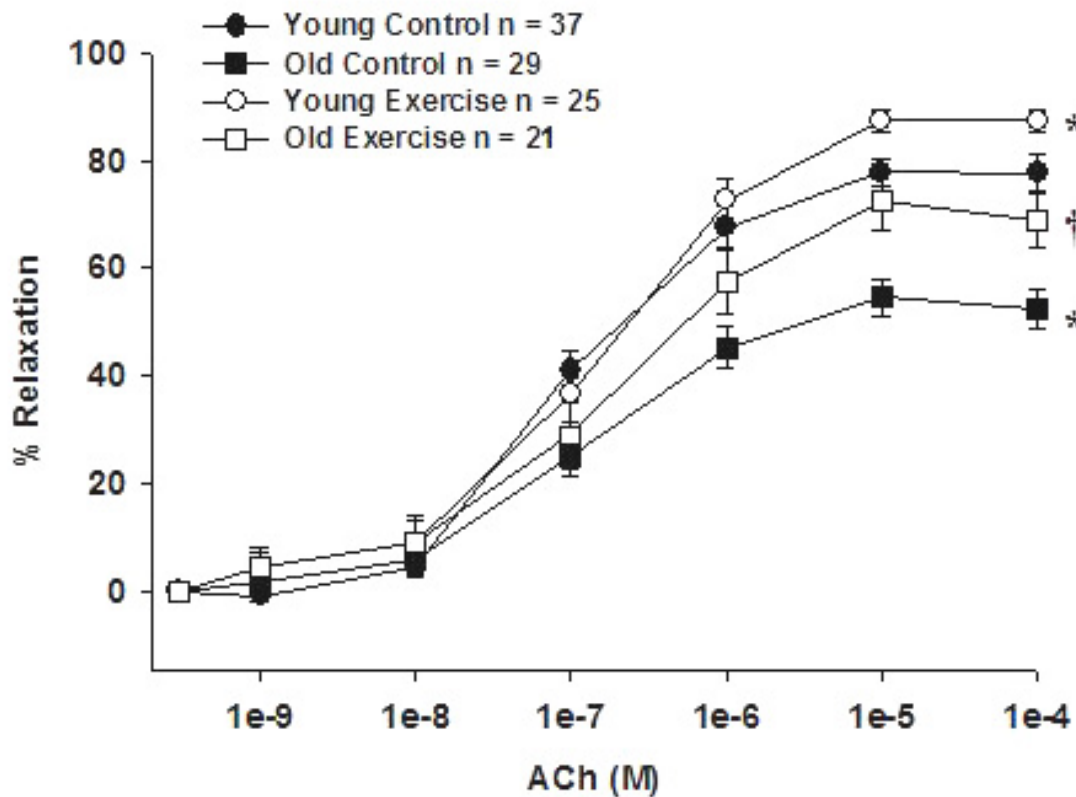


Figure 2.1. ACh-induced vasodilation in soleus muscle arterioles from young and old, sedentary and exercise-trained rats to acetylcholine (ACh). Age significantly reduced vasodilator responses to ACh in soleus muscle arterioles. Exercise training restored vasodilator responses to ACh in soleus muscle arterioles from old rats. Diameter changes in response to increasing intraluminal pressure in soleus muscle arterioles. * $P < 0.05$ vs. YSED and † $P < 0.05$ vs. OSED.

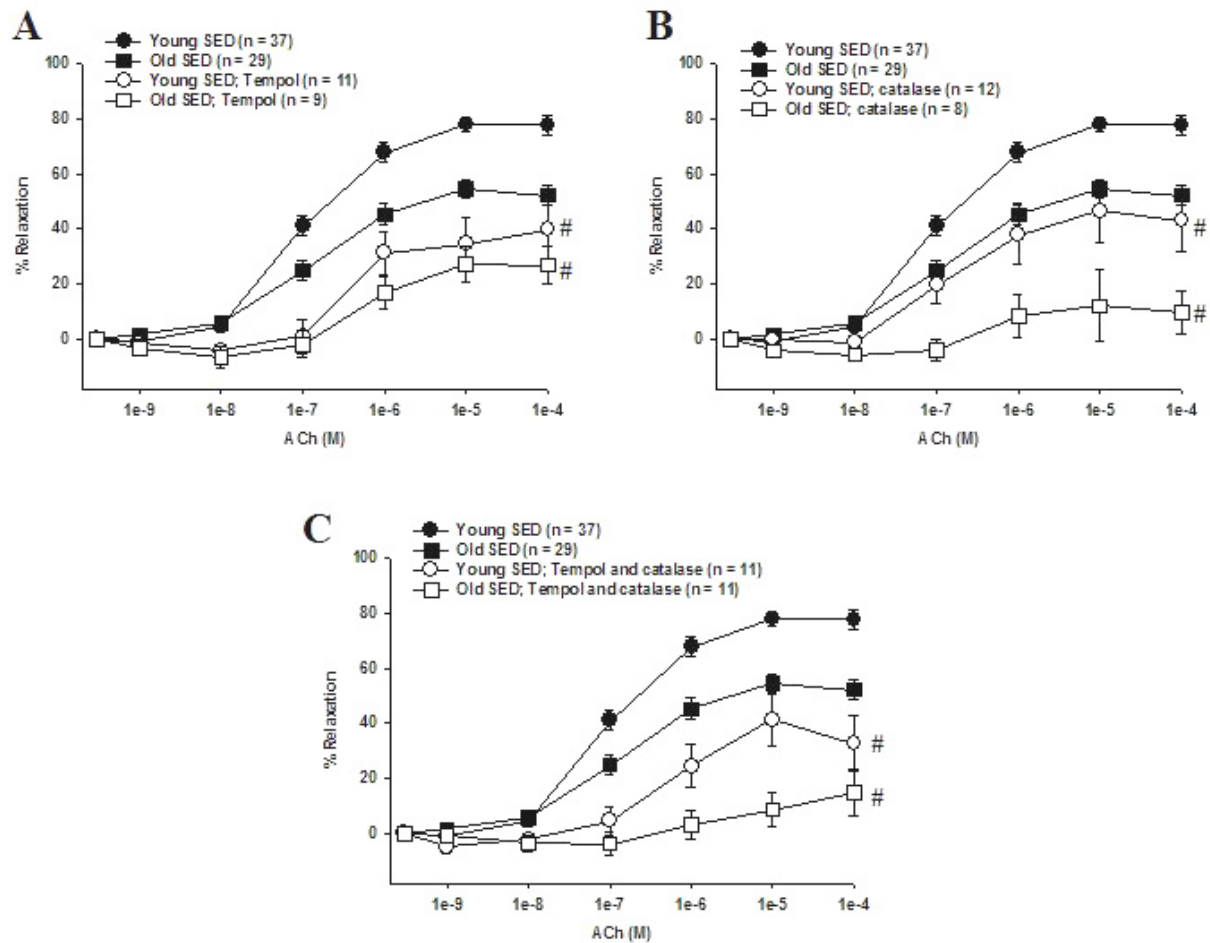


Figure 2.2. Effect of Tempol and catalase on ACh-induced vasodilation of soleus muscle arterioles from young and old sedentary rats. (A) Tempol inhibited the vasodilator response to ACh in arterioles from all groups and abolished the age differences in ACh-induced vasodilation. (B) Catalase inhibited the vasodilator response to ACh-induced in arterioles from all rats, however the age difference in ACh-induced vasodilation remained. (C) Combination of Tempol and catalase inhibited vasodilator responses to ACh in arterioles from all rats and abolished the age differences in ACh-induced vasodilation. Data are presented as means \pm SE. # $P < 0.05$ Inhibitor + ACh vs. ACh alone.

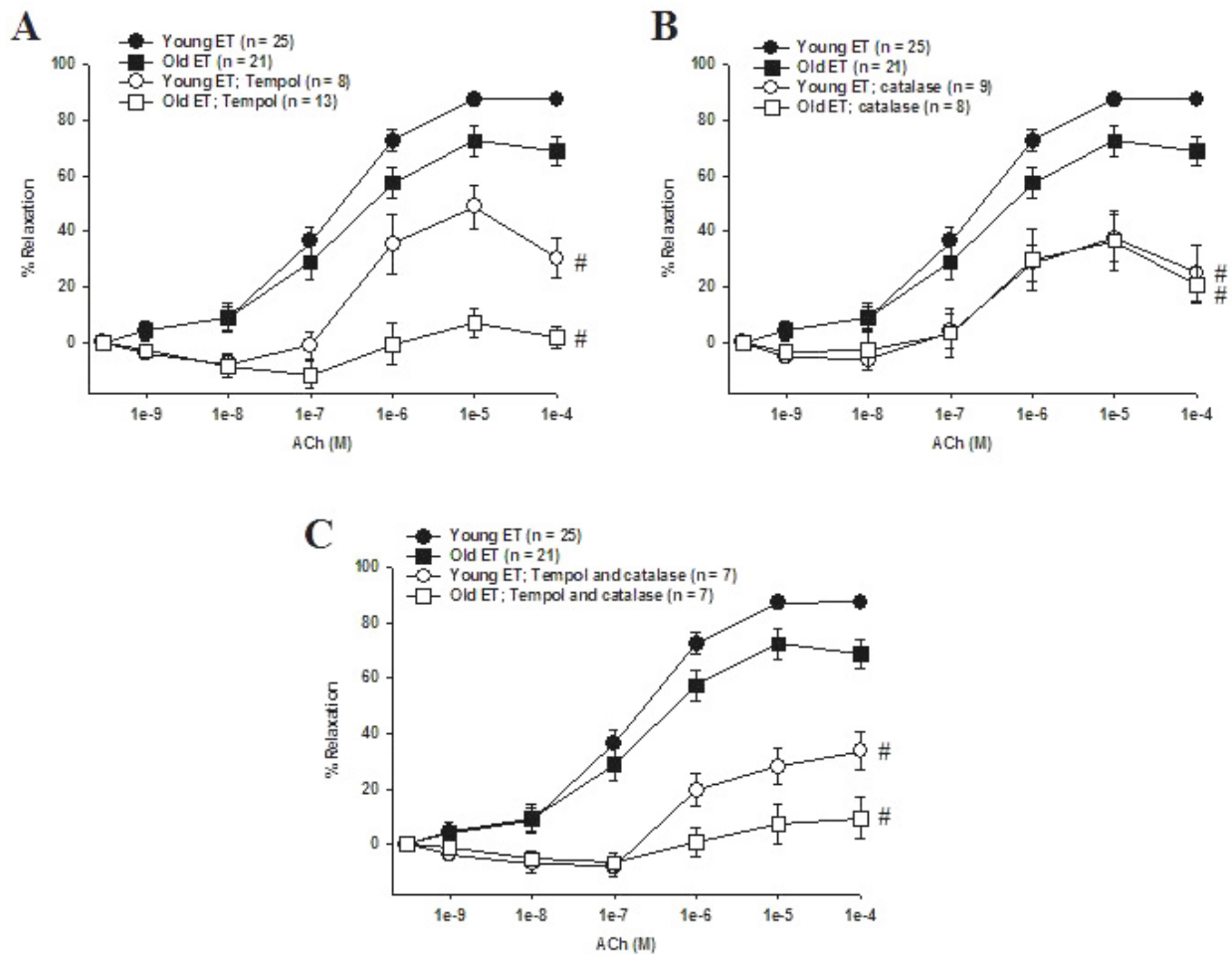


Figure 2.3. Effect of Tempol and catalase on ACh-induced vasodilation of soleus muscle arterioles from young and old exercise-trained rats. (A) Tempol inhibited the vasodilator response to ACh in arterioles from all groups; however age differences developed in ACh-induced vasodilation. (B) Catalase inhibited the vasodilator response to ACh-induced in arterioles from all rats. (C) Combination of Tempol and catalase inhibited vasodilator responses to ACh in arterioles from all rats. # $P < 0.05$ Inhibitor + ACh vs. ACh alone.

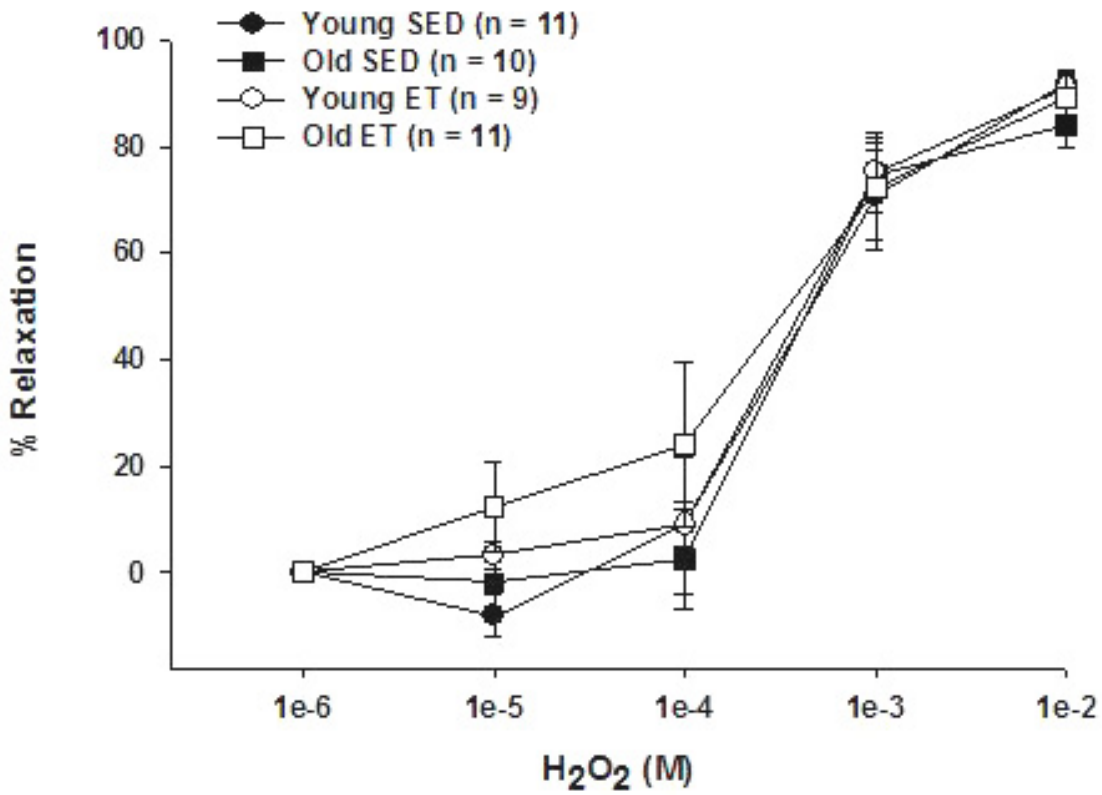


Figure 2.4. H₂O₂-induced vasodilation in young and old, sedentary and exercise-trained rats. Vasodilation to exogenous H₂O₂ was preserved with age and was unchanged by exercise training.

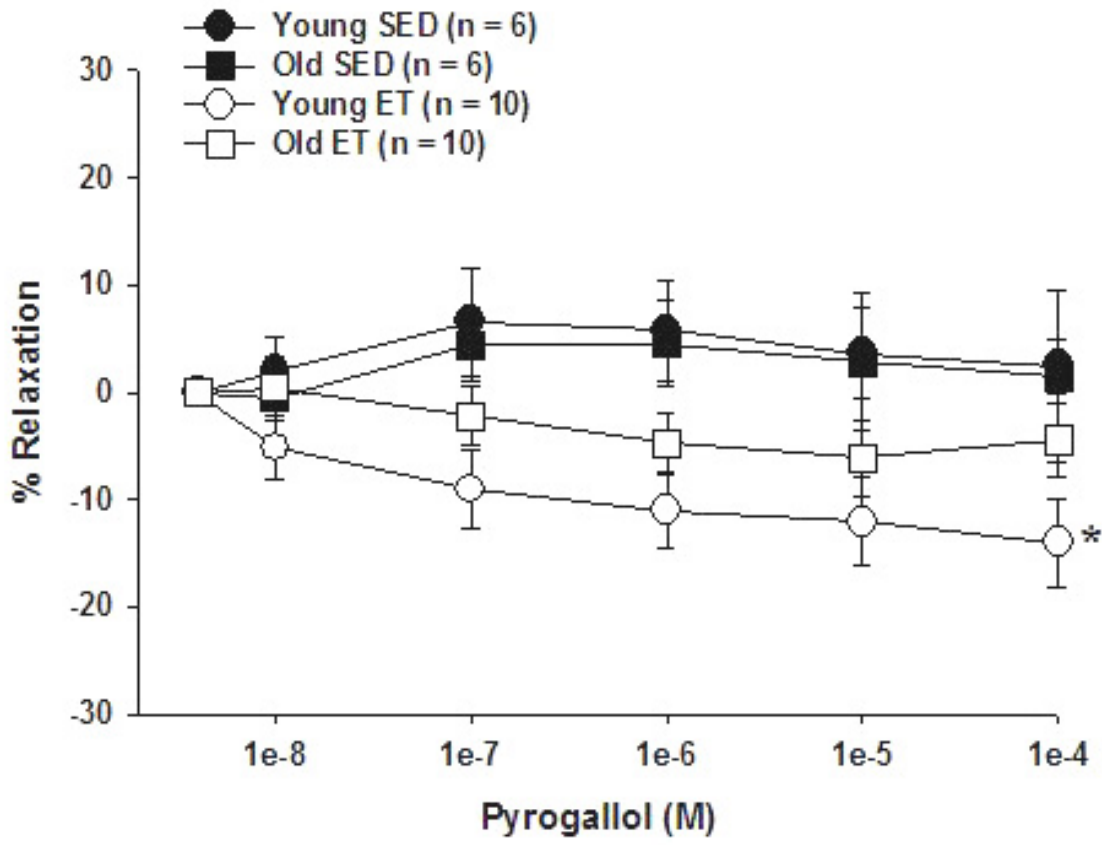


Figure 2.5. O₂⁻-induced vasoactive characteristics in young and old, sedentary and exercise-trained rats. Pyrogallol induced vasoconstriction in old ET rats. **P* < 0.05 OSED vs. OET.

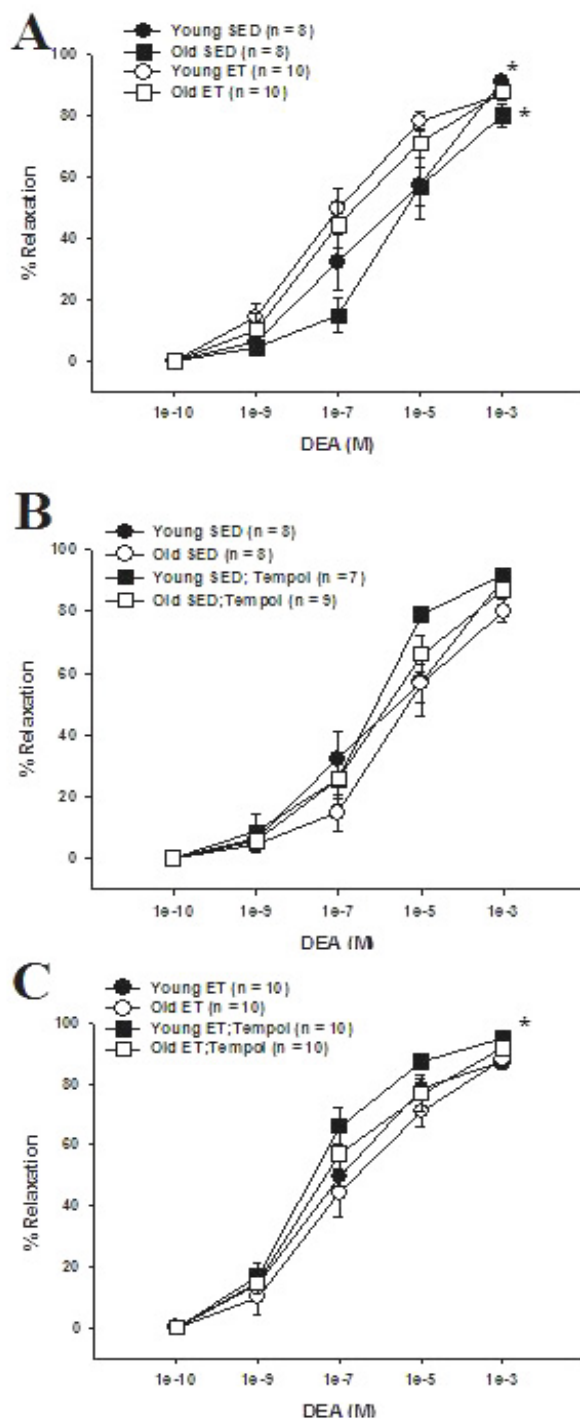


Figure 2.6. Endothelium-independent vasodilation to Dea-NONOate in soleus muscle arterioles from young and old, sedentary and exercise-trained rats. (A) Vasodilation to Dea-NONOate was similar in soleus muscle arterioles from young and old SED rats; however, exercise training increased vasodilatory responses to Dea-NONOate in both young and old rats. (B) Scavenging of superoxide with Tempol had no effect on vasodilator responses to Dea-NONOate in soleus muscle arterioles from young and old SED rats. (C) Scavenging of superoxide with Tempol had no effect on vasodilator responses to Dea-NONOate in soleus muscle arterioles from young and old ET rats. * $P < 0.05$ SED vs. ET.

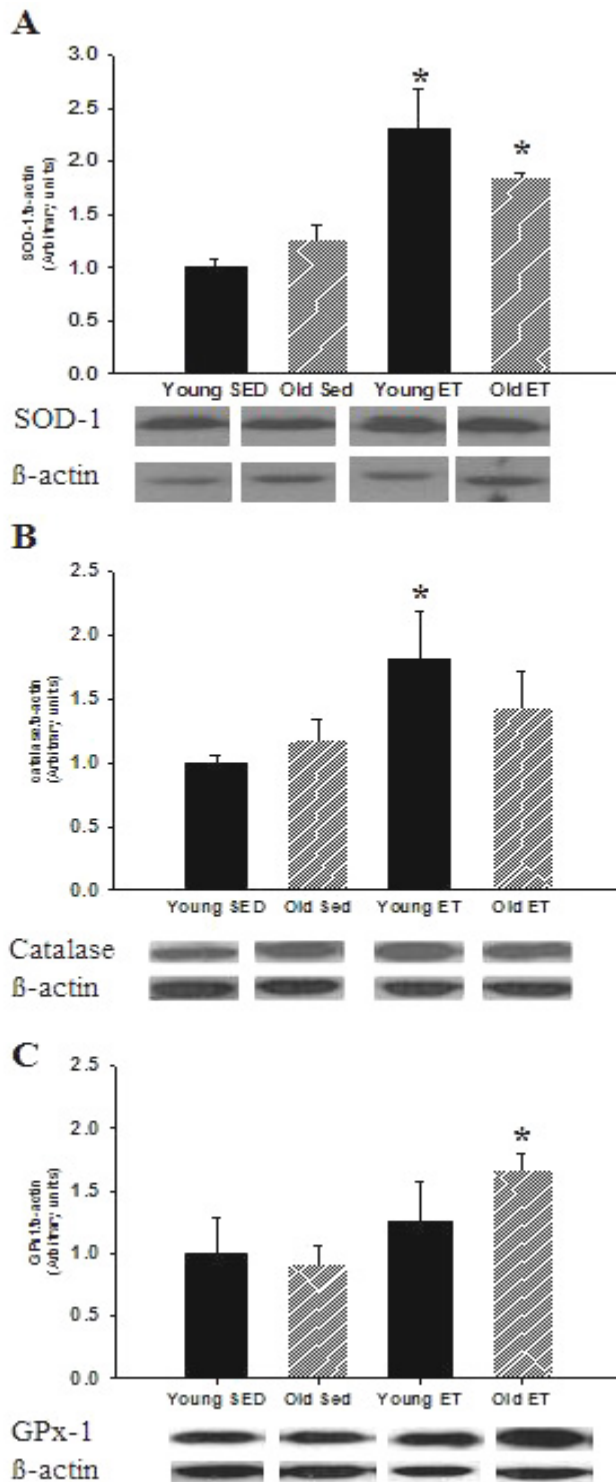


Figure 2.7. (A) Superoxide dismutase (SOD-1), (B) catalase, and (C) glutathione peroxidase (GPx1) protein content in soleus muscle arterioles of young and old, sedentary and exercise-trained rats. * $P < 0.05$ indicates exercise training effect.

	Young SED (n = 37)	Young ET (n = 28)	Old SED (n = 29)	Old ET (n = 21)
Animal Characteristics				
Body Wt (g)	370 ± 4	345 ± 4†	429 ± 6*	378 ± 6†
Soleus muscle wt (mg)	153.0 ± 3.1	156.8 ± 4.2	163.7 ± 4.0*	171.8 ± 5.2
Soleus wt/Body wt (mg/g)	.41 ± 0.01	.45 ± 0.01*	.38 ± 0.01*	.45 ± 0.01†
Vessel Characteristics				
Maximal Diameter (µm)	128 ± 4	127 ± 5	120 ± 4	121 ± 5
Spontaneous tone (%)	50 ± 2 (n = 37)	51 ± 2 (n = 28)	54 ± 2 (n = 29)	56 ± 2 (n = 21)
with Tempol	54 ± 4 (n = 11)	47 ± 4 (n = 9)	60 ± 4 (n = 9)	60 ± 3 (n = 13)
with Tempol + Catalase	50 ± 4 (n = 11)	51 ± 5 (n = 8)	51 ± 3 (n = 11)	51 ± 2 (n = 8)
with Catalase	52 ± 3 (n = 12)	51 ± 5 (n = 9)	57 ± 4 (n = 8)	56 ± 6 (n = 9)

Table 2.1. Animal and vessel characteristics of young and old rat, sedentary and exercise trained rats. Maximal diameter was recorded in Ca²⁺-free physiological saline solution with 100µM sodium nitroprusside. Tone (%) = [(maximal diameter – diameter with tone)/maximal diameter] × 100. **P* < 0.05 indicates age effect. †*P* < 0.05 indicates exercise training effect.

Discussion

This study confirms our previous report that ACh-induced vasodilation declines with age and is restored by exercise training (122). Several new findings from this study indicate that ROS are important mediators of age- and training-induced adaptations of endothelium-dependent vasodilation in skeletal muscle arterioles. First, H₂O₂ is necessary for endothelium-dependent vasodilation in skeletal muscle arterioles. Second, the dependence on ACh-induced vasodilation on H₂O₂ increases with age. Third, exercise training improves endothelium-dependent vasodilation in old rats, in part, by decreasing dependence on H₂O₂. Fourth, the sensitivity of ACh-induced vasodilation to exogenous SOD changes with age and by exercise training. Thus, a tight balance between enzymatic production of O₂⁻ and H₂O₂ must be maintained for robust endothelium-dependent vasodilation to occur.

It is becoming clear that O₂⁻ and O₂⁻-derived ROS are important signaling molecules involved in the regulation of physiological function (41). More specifically, H₂O₂ generated by the dismutation of O₂⁻ is emerging as an important vasodilator and signaling molecule in the vasculature (21). O₂⁻ is derived from several sources in the endothelium including eNOS (42), mitochondria (77), xanthine oxidases (52) and NADPH oxidases (15). Endothelium-derived H₂O₂ contributes to vasodilation in isolated coronary arterioles (72). In skeletal muscle arterioles H₂O₂ has been shown to produce biphasic responses; low concentrations of H₂O₂ (10⁻⁶ to 3 X 10⁻⁵) induce vasoconstriction whereas higher concentrations elicit vasodilation (27). Similarly, in pulmonary (13), skeletal muscle (27), cerebral, (139) and mesenteric (84) vascular beds exogenous H₂O₂ causes vasodilation. Thus, vasodilation elicited by authentic H₂O₂ was preserved with age and was unchanged by exercise training in skeletal muscle arterioles (Fig. 2.4). H₂O₂ has been labeled as endothelium-derived hyperpolarization factor (EDHF) because it is produced by the endothelium and elicits vascular smooth muscle relaxation through activation of Ca²⁺ dependent

K^+ (K_{Ca}) channel activation (82, 85). Our results do not indicate the mechanism by which H_2O_2 produced vasodilation in skeletal muscle arterioles; however, our findings that neither age or exercise training altered responsiveness of soleus muscle arterioles to exogenous H_2O_2 suggests that adaptations in H_2O_2 signaling are confined to the endothelium and occur due to changes in endogenous production and/or generation of H_2O_2 .

In the present study, the dependence of ACh-induced vasodilation on H_2O_2 was more pronounced in old SED rats (Fig. 2.2B). This increased dependence on H_2O_2 was reversed by exercise training in arterioles from old rats (Fig. 2.3B). Our previous results suggest that age reduces NO-mediated dilation, whereas exercise training promotes endothelial signaling through NO (122). Thus our current results suggest that endothelial H_2O_2 signaling increases with age as NO-mediated dilation declines. In contrast, exercise training appears to promote reversal to a younger vasodilatory system, which requires less dependence on H_2O_2 signaling, and greater dependence on NO signaling.

Age-related differences in ACh-induced vasodilation were eliminated by scavenging of O_2^- with the exogenous SOD mimetic, Tempol (Fig. 2.2A) indicating that alterations of O_2^- signaling contribute to age-associated impairment in ACh-induced vasodilation. The finding that Tempol reduced vasodilation in skeletal muscle arterioles from all groups of rats is surprising; however, Marvar *et al.* reported that locally generated ROS actually contribute to functional dilation (83). We propose that scavenging of O_2^- in SED rats reduces vasodilation by two possible mechanisms. First, O_2^- may act as a vasodilatory agent, as reported in cerebral arterioles of mice (140). Second, SOD has the capability of producing OH^- in the presence of transition catalytic metals (i.e., iron), as observed in aged rats (12). In coronary arterioles, iron chelation with deferoxamine, reverses OH^- -mediated vasoconstriction (131). If the addition of exogenous SOD increases H_2O_2 production dramatically, this could overwhelm the endogenous catalase system, leading to rapid production of OH^- , and vasoconstriction.

In the present study, H_2O_2 -generated by Tempol was catalyzed to H_2O with the addition of catalase, thus scavenging both O_2^- and O_2^- -derived H_2O_2 . This simultaneous scavenging of both O_2^- and H_2O_2 eliminated ACh-induced vasodilation in old SED rats, supporting our hypothesis that even though ACh-induced vasodilation is already diminished with age, O_2^- -derived ROS are necessary for vasodilation in old SED rats. In young SED and ET rats, some dilation to ACh remained during simultaneous scavenging of both O_2^- and H_2O_2 indicating the contribution of ROS-independent signaling in arterioles from these young rats.

The restoration of endothelium-dependent vasodilation observed in old rats subjected to exercise training (Fig. 2.1) confirms our previous study (122). O_2^- generation and oxidant stress increase with exercise training (4, 5, 30). This increase in oxidant stress probably contributes to enhanced O_2^- and O_2^- -derived ROS signaling in the endothelium of skeletal muscle arterioles of exercise trained rats (Fig. 2.3). The enhanced regulation of O_2^- and O_2^- -derived ROS may depend on protein levels and activities of anti-oxidant enzymes. For example, Rush et al. demonstrated in aortic endothelial cells (AEC) from young pigs that SOD-1 protein abundance and activity increased with training but had no effect on catalase content (113). In our present study, exercise training increased SOD-1 protein levels in skeletal muscle arterioles from young and old rats (Fig. 2.7A) indicating that more H_2O_2 was produced in the vasculature of exercise-trained rats. Interestingly, exercise training increased catalase in the young rats only (Fig. 2.7B); however, exercise training increased GPx1 in the old rats (Fig 2.7C). Similar to GPx, catalase catalyzes the breakdown of H_2O_2 ; however, GPx has a higher affinity for H_2O_2 at low concentrations ($K_m = 1 \mu\text{M}$) than catalase ($K_m = 1 \text{ mM}$), indicating that GPx is a less effective antioxidant (90). These age-specific adaptations to exercise training may contribute to the increased dependence on H_2O_2 as a vasodilator in arterioles from old rats.

In skeletal muscle arterioles, O_2^- may have direct vasoactive effects. To test this hypothesis, we determined the vasoreactivity of skeletal muscle arterioles to increasing concentrations of pyrogallol, which produces O_2^- by auto-oxidation (81). Pyrogallol caused slight vasoconstriction only in young ET rats (Fig. 2.5). Addition of exogenous O_2^- has no effect in SED rats (Fig. 2.5) indicating that O_2^- is not acting directly to cause vasodilation of vascular smooth muscle, but as a signaling molecule that modulates endothelium-dependent vasodilation. Removal of O_2^- using Tempol impairs vasodilation, whereas addition of exogenous O_2^- should increase dilation. Pyrogallol did not have any vasodilatory effects; however, it is possible that O_2^- produced by pyrogallol did not permeate the endothelial cell membrane, and thus, did not alter intracellular signaling in the endothelium. Therefore, the inhibition of vasodilation produced by Tempol occurred predominately because of dismutation of O_2^- to H_2O_2 ; however, direct manipulation of intracellular levels of O_2^- would be necessary to determine precisely the endothelial signaling pathways that are directly modulated by O_2^- . These data confirm our results obtained in experiments in which Dea-NONOate and Tempol were applied simultaneously. Tempol did not alter responsiveness to Dea-NONOate except in young ET rats, indicating that O_2^- signaling occurs within the endothelium, possibly through O_2^- -derived H_2O_2 and/or OH^- production, or through scavenging of intracellular NO. Therefore, the reduced NO bioavailability that occurred with age in skeletal muscle arterioles, was not the result of increased scavenging of NO by extracellular O_2^- .

A limitation of the present study is that we did not directly measure O_2^- and/or H_2O_2 during manipulation of SOD and/or catalase activity. H_2O_2 can elicit biphasic responses on skeletal muscle arterioles (27), thus the final effect of manipulation of either SOD or catalase activity may depend on the concentration of H_2O_2 generated under these conditions. However, alterations in protein levels measured do correlate with increased reliance on H_2O_2 signaling with age, and a reversal of H_2O_2 dependence in the endothelium of skeletal muscle arterioles.

In conclusion, the current study implicates O_2^- and O_2^- -derived ROS (H_2O_2) as necessary signaling molecules required for endothelium-dependent vasodilation in soleus muscle arterioles. The dependence of ACh-induced vasodilation on H_2O_2 increases with age and decreases with exercise training. Furthermore, exercise training contributes to regulation of the relative production of O_2^- and H_2O_2 , maintaining endothelium-dependent vasodilation in skeletal muscle arterioles.

Chapter IV

General Discussion

The goal of this project was to better understand the mechanisms by which exercise training reverses age-related impairment of endothelium-dependent vasodilation in skeletal muscle arterioles. Both studies presented confirm our previous work, which indicated that endothelium-dependent vasodilation is reduced in skeletal muscle arterioles from old rats. Exercise training restores endothelium-dependent vasodilation in arterioles from old rats and improves endothelium-dependent vasodilation in skeletal muscle arterioles from young rats.

The first study demonstrated that reductions in flow-induced vasodilation are accompanied by decreases in BH₄ levels in skeletal muscle arterioles from old rats. Furthermore, the limited availability of BH₄ in arterioles from old SED rats occurred concomitantly to a subsequent decrease in NO signaling and an increase in eNOS-derived O₂⁻ generation, suggesting a role for eNOS uncoupling. Exercise training restored BH₄ levels and improved flow-induced NO production in arterioles from old rats. Exercise training increased both NO and ROS-mediated signaling in skeletal muscle arterioles, suggesting that the exercise training-induced enhancement of flow-induced vasodilation in skeletal muscle arterioles involves a balance between NO and O₂⁻-derived ROS. Thus, the beneficial effects of exercise training in the skeletal muscle resistance vasculature involves more than improvement of NO bioavailability.

In our second study, we found that O₂⁻ and O₂⁻-derived ROS (H₂O₂) are required for endothelium-dependent vasodilation in skeletal muscle arterioles. The dependence of ACh-induced vasodilation on H₂O₂ increases with age and decreases with exercise training. Thus, exercise training contributed to the regulation of the relative production of O₂⁻ and H₂O₂ and that a balance between these ROS must be maintained for robust endothelium-dependent vasodilation to occur in skeletal muscle arterioles.

Taken together, these studies demonstrate that both NO and H₂O₂ are important signaling molecules necessary for mediating endothelium-dependent vasodilation in skeletal muscle arterioles. With advancing age, the dependence of endothelial function on H₂O₂ increases as NO signaling declines in skeletal muscle arterioles. The existence of a tight balance between NO and H₂O₂ probably exists and is altered with age and with exercise training.

H₂O₂ can potentially modulate NO by a variety of mechanisms to increase NO production. For example, H₂O₂ activates eNOS through a series of coordinated phosphorylation and dephosphorylation steps through a phosphoinositide 3-kinase-dependent signaling pathway (133). Additionally, H₂O₂ stimulates GTPCH-1 protein, which is the rate-limiting enzyme for *de novo* synthesis of BH₄ and elicits increases in both BH₄ synthesis and eNOS activity in endothelial cells (118, 119).

In conclusion, impaired endothelium-dependent vasodilation is a risk factor for development of cardiovascular disease in aged individuals. Exercise training augments endothelium-dependent vasodilation, and thus reduces cardiovascular disease risk. However, the underlying mechanisms by which exercise training improves endothelium-dependent vasodilation have not been fully elucidated. The data in this dissertation are the first to demonstrate a potential mechanism by which exercise training restores age-associated impairments in endothelium-dependent vasodilation, by restoring BH₄ levels and increasing both NO- and ROS-mediated signaling in skeletal muscle microcirculation.

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Curriculum Vitae

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Publications

Peterson JM, Bryner RW, Sindler A, Frisbee JC, Alway SE, Mitochondrial apoptotic signaling is elevated in cardiac but not skeletal muscle in the obese Zucker rat and is reduced with aerobic exercise. *J Appl Physiol*, 2008. 105(6):1934-43.

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Manuscripts in Review and/or Preparation

Sindler, A.L., Reyes, R.A., Muller-Delp, J.M. Age and exercise training alter signaling through reactive oxygen species in the endothelium of skeletal muscle arterioles (in preparation)

Sindler, A.L., Delp, M.D., Reyes, R., Wu, G., Muller-Delp, J.M. Effects of aging and exercise training on eNOS uncoupling in skeletal muscle resistance arterioles (in preparation)

Kang, L.S., Kim S.J., Sindler, A.L., Dick, G.M., and Muller-Delp, J.M. Contribution of K⁺ Channels to the Myogenic Response in Skeletal Muscle Arterioles: Effects of Aging and Fiber Type (in preparation)

Sindler, A.L., Hileman, S.M., Peterson, J.M, Bryner, R.W., Frisbee, J.C. and Muller-Delp, J.M. Effect of Exercise Training on Adiponectin and Adiponectin Receptors in Zucker Rats. *Med Sci Sports Exerc*. 2008 (in preparation)1. Heart Disease and Stroke Statistics - 2007 Update At-a-Glance. Dallas, Texas: American Heart Association, 2006.

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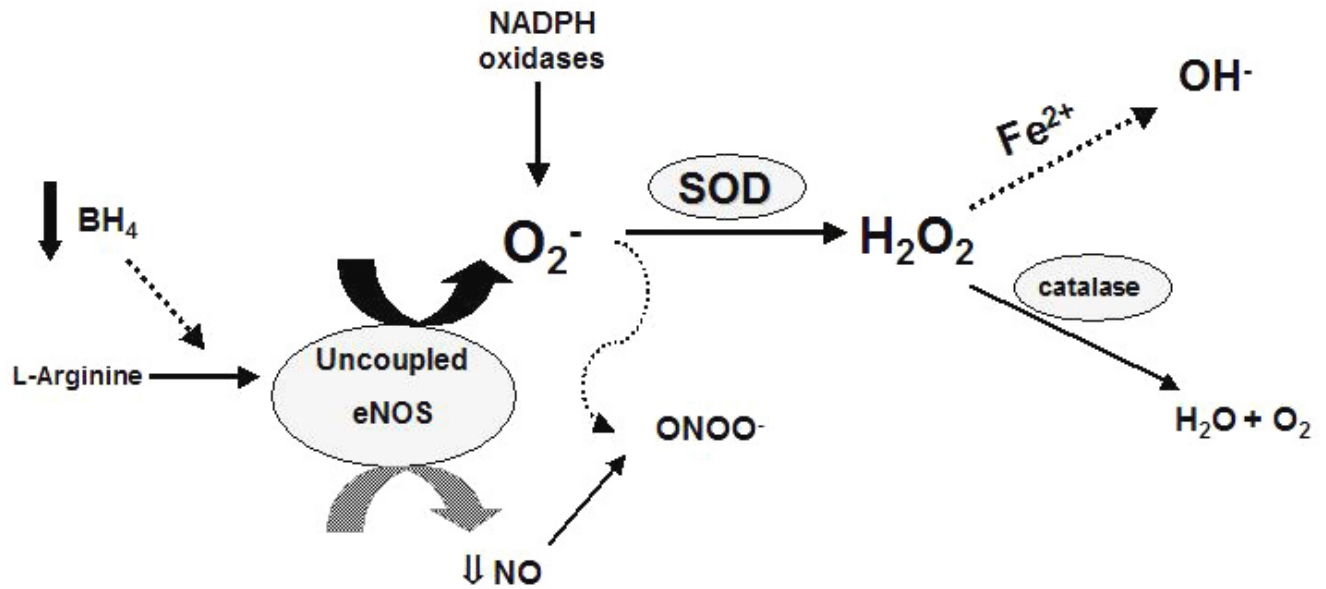
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Appendix



Proposed effects of aging 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^\cdot . H_2O_2 is converted to H_2O and O_2 in the presence of catalase.