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Aging, Habitual Exercise, and Vascular Ischemia-Reperfusion Injury

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Dedication

To my husband, Justin, and to my family: my mother, my father, and my brother.

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Aging, Habitual Exercise, and Vascular Ischemia-Reperfusion Injury

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Ischemia-reperfusion (IR) injury occurs during myocardial infarction and during some cardiovascular surgeries. Animal studies support the role of endurance exercise training in preventing myocardial IR injury and coronary endothelial dysfunction. In human and animal studies, habitual exercise has been shown to attenuate endothelial dysfunction caused by aging and disease. It is unknown; however, if exercise can protect against vascular IR injury in humans and if so, whether these effects persist with advancing age. Using 20 minutes of forearm ischemia and the response of the brachial artery as a noninvasive surrogate model for the heart, the association between the mode of exercise training (endurance versus resistance) and vascular IR injury was examined in young healthy adults in the first study. Endothelial function, as measured by flow-mediated dilation (FMD) in the brachial artery, decreased significantly after forearm ischemia, suggesting that this noninvasive model of the heart produces significant and measureable vascular injury. These measures returned to baseline levels within 30 minutes following ischemia, illustrating the transient nature of this form of IR injury. The magnitude of injury and recovery from ischemia were not significantly different

among young sedentary, endurance-trained, and resistance-trained subjects, suggesting that exercise training is not associated with protection from vascular IR injury in a young, healthy population.

In the second study, the association between aging, endurance exercise training, and vascular IR injury was studied. Twenty minutes of forearm ischemia was associated with a transient fall in brachial FMD in young and older sedentary and endurance-trained subjects. Young subjects recovered more quickly from IR injury than older subjects. Within 30 minutes of injury, the endothelial function of the young group was back to baseline while blunted endothelial function persisted in older subjects for greater than 45 minutes after injury. There was no association between endurance exercise training and enhanced recovery from IR injury. These findings suggest that aging is associated with delayed recovery from vascular IR injury and that endurance training does not appear to modulate the vascular IR injury responses.

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Chapter 1: General Introduction

Of the major causes of mortality in the United States, cardiovascular disease ranks first (306, 365, 405), and each day, approximately 2,400 Americans die from cardiovascular disease (6). Along with lifestyle habits and genetics, advancing age is an important risk factor for cardiovascular disease. With the elderly population and obesity growing, the incidence of cardiovascular disease will continue to rise (31), making the prevention and treatment of cardiovascular diseases an urgent topic of research. Germane to this research is the need for a greater understanding of the etiology and pathogenesis of myocardial infarction, which occurs when blood flow to the heart and vasculature is disrupted, producing tissue damage through a process called ischemia-reperfusion (IR) injury. This IR injury also occurs during cardiovascular surgery to prevent or treat cardiovascular disease. In particular, traditional coronary artery bypass graft surgery requires cardiopulmonary bypass, and the introduction of arterial or venous grafts, rendering the heart and blood vessels ischemic for brief periods of time (53).

Arterial endothelial dysfunction is an important mechanism underlying tissue damage induced by ischemia-reperfusion, and endothelial dysfunction has been shown to be an independent predictor of future cardiovascular events (41, 58, 193, 260, 263, 275). With advancing age and cardiovascular disease, endothelial function declines, but habitual exercise preserves endothelial function, perhaps by increasing nitric oxide bioavailability, lowering oxidative stress, and reducing inflammation (140, 155, 218, 309). In animals, aerobic exercise training also protects against myocardial IR injury (295) and attenuates endothelial dysfunction from disease in the vasculature (178). However, it is unknown if exercise training can protect against myocardial and vascular

IR injury in humans, and safety issues hinder the direct study of this topic. In the following studies, we use a "surrogate model of the heart", inducing 20 minutes of limb ischemia and following the endothelial function of the brachial artery to address these questions. The first study examined the association between the mode of exercise training (endurance versus resistance exercise) and vascular IR injury in young adults. In the second study, the association between aging, endurance training, and vascular IR injury was studied. If exercise training can improve nitric oxide bioavailability, lower oxidative stress, and reduce inflammation, which should theoretically attenuate endothelial dysfunction after IR injury, it may become an important non-pharmacological strategy for the prevention and treatment of cardiovascular disease. In addition, because the majority of cardiovascular events occur in older adults, the interaction of exercise training with age on vascular recovery is clinically significant. By uncovering the mechanisms underlying the possible cardiovascular protection afforded by exercise training in humans, scientific understanding of cardiovascular disease pathology may be enhanced, leading to new treatment strategies.

Chapter 2: Review of Literature

Numerous studies have shown that short-term and long-term aerobic exercise training of moderate and high intensity protects the myocardium against ischemia-reperfusion (IR) injury in animals (36, 37, 45, 47, 89, 295, 336). Suggested mechanisms by which aerobic exercise training may provide cardioprotection against IR injury, especially late phase cardioprotection (47, 336, 337), include alterations in cardiac metabolism, calcium handling, oxidative stress, inflammation, and nitric oxide bioavailability (186). During ischemia, energy depletion and reactive oxygen species production occurs from inadequate blood flow to tissues. With reperfusion, re-entry of the blood into ischemic tissues causes additional damage through calcium loading, inflammation, and a surge of free radical production (167). Within the vasculature, exercise training may also increase nitric oxide bioavailability, directly mediating endothelial function through enhanced vasodilation. In addition, nitric oxide's interaction with potassium channels may alter cardiac and vascular metabolism and calcium handling, and may indirectly lower oxidative stress and reduce inflammation. Along with its effects on nitric oxide, exercise training may also lower oxidative stress and inflammation independent of nitric oxide, which may decrease endothelial cell necrosis and apoptosis following ischemia and reperfusion. In the following review, exercise-induced protection from IR injury in the animal heart and vasculature will be discussed. Mechanisms by which aerobic exercise training may increase nitric oxide bioavailability, lower oxidative stress, and lower inflammation in animals and humans will be examined and its implications for human cardiovascular health will be explored.

ANIMAL STUDIES: NITRIC OXIDE, EXERCISE TRAINING, AND MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

A recent review of ischemia-reperfusion (IR) injury in the myocardium found that a majority of in vitro and in vivo animal studies showed that endogenous and exogenous administration of nitric oxide provides cardioprotection (27). In addition, treatments such as ischemic preconditioning (393, 394) and pharmacological preconditioning (27) enhance late phase cardioprotection against IR injury (12 hours to four days after treatment) by acting both as a trigger and mediator of nitric oxide production. During the first 12 to 24 hours after preconditioning, endothelial nitric oxide synthase (eNOS) is stimulated to produce nitric oxide. The resulting increase in eNOS-produced nitric oxide stimulates an increased expression and activity of inducible nitric oxide synthase (iNOS) during the 24 to 72 hours after preconditioning, which also increases nitric oxide bioavailability (28). The importance of nitric oxide in attenuating IR injury in the heart has also been demonstrated in animal studies manipulating eNOS expression and activity. Mice genetically engineered to be deficient in eNOS had greater damage (187) while mice over-expressing eNOS had less damage following cardiac IR injury (188). Also, the administration of statins, a drug that increases the stability of eNOS mRNA and activates protein kinase Akt, decreased infarct size caused by IR in animal hearts (389). By increasing the phosphorylation and expression of eNOS, statins increase the bioavailability of nitric oxide through enhanced nitric oxide production (389).

With aerobic exercise training, nitric oxide may play a large role in cardioprotection against IR injury in animals. For example, after short-term exercise training on a treadmill, trained mice had smaller myocardial infarct size compared with control mice, but this protective effect disappeared when a NOS inhibitor was administered (144). In addition, when eNOS-deficient (knockout) mice were exercised

using the same treadmill protocol, exercise-induced protection was abolished, suggesting that exercise-stimulated nitric oxide plays a protective role against IR injury (144). Moreover, an abstract published five years later by the same research group corroborates these findings. Once accustomed to treadmill exercise, mice were exercise-trained for two days. Half an hour after exercise, the levels of nitrite/nitrate, phosphorylated eNOS, PKC-activity, and PKC translocation were higher compared with non-exercised wild-type and eNOS knockout hearts. Additionally, 30 minutes of ischemia and 24 hours of reperfusion caused significantly greater myocardial damage in the control groups compared with the exercise group (145). Administration of a NOS inhibitor and a protein kinase C inhibitor (chelerythrine) abolished the protective effect of exercise. These findings support the role of exercise in protecting the mouse heart from IR injury through eNOS-produced nitric oxide and eNOS activation of protein kinase C (145, 394). By activating protein kinase C, transcriptional activation of the iNOS gene leads to additional nitric oxide production (189). A canine model also supports the role of nitric oxide in exercise-induced cardioprotection against IR injury. Dogs exposed to 21 minutes of treadmill exercise had less dangerous arrhythmias and ECG changes in addition to preserved baroreflex sensitivity and higher levels of cardiac iNOS activity compared with controls after IR injury (13). When aminoguanidine (a NOS inhibitor) was administered, the protective effects of exercise were abolished. Also, after two days of exercise in a hypothermal environment, rats had higher eNOS expression than sedentary controls (361).

Other animal studies suggest that nitric oxide may not play a primary role in exercise-mediated cardioprotection (336). A study in rats found that the administration of a global NOS inhibitor, N-nitro-L-arginine methyl ester hydrochloride (L-NAME), did not diminish the cardioprotective effects of two days of exercise to IR injury (361). A

follow-up study using a longer training period found that L-NAME did not abolish the cardioprotective effects of four weeks of exercise training on IR injury in rats (336). Similarly, eight weeks of treadmill running was associated with decreased levels of eNOS in female rat hearts and no change in eNOS levels in male rat hearts (367). One possible explanation for the cardioprotective benefits of exercise training even with NOS inhibition or without changes in eNOS protein expression is that nitric oxide can be produced by pathways other than NOS (244). The NOS-independent pathway of nitric oxide production using nitrite by myoglobin, hemoglobin, and/or xanthine oxidoreductase may be especially important during IR injury because ischemia produces a low oxygen, acidic environment, which activates these pathways while simultaneously inhibiting eNOS production of nitric oxide (127, 244). Indeed, these studies did not measure nitric oxide breakdown products within the tissues.

ANIMAL STUDIES: ENDOTHELIAL FUNCTION, EXERCISE TRAINING, AND VASCULAR ISCHEMIA-REPERFUSION INJURY

With regard to the vascular tissue itself, animal studies involving exercise training, transient ischemia, and the effects on the endothelium are few. Ischemia-reperfusion (IR) injury in many studies involves prolonged ischemia of days and weeks (heart failure models) or a focus on damage to the perfused organ, not the artery itself. Only two animal studies are known to have examined the effects of exercise training before IR injury in arteries (83, 348). Female sedentary rats and age-matched rats that ran five days a week for ten weeks, peaking at 60 minutes a day at 26.8 meters per minute, were exposed to short-term ischemia (1 x 5 minutes) or long-term ischemia (3 x 15 minutes with five to 10 minutes recovery) by left coronary artery occlusion in situ (348). Within ten minutes of the last bout of ischemia, coronary resistance arteries were isolated, mounted, precontracted with potassium chloride (KCl), and dose-response curves to acetylcholine, U-46619 (prostaglandin H2-thromboxane A2 receptor agonist), endothelin-1, and sodium nitroprusside were determined. Vasodilatory responses to acetylcholine or sodium nitroprusside were not different between sedentary and exercise trained rats after short or long-term ischemia, but vasoconstriction by U-46619 and endothelin-1 was greater in sedentary compared with trained rats after long-term ischemia, but not after short-term ischemia. In exercise trained rats that were not exposed to IR injury, but underwent sham-surgery, vasorelaxation responses to sodium nitroprusside were lower at most concentrations and one concentration of U-46619 than sedentary, sham-operated rats. No differences were seen in vasorelaxation responses to acetylcholine or endothelin-1.

Another study in male rats found similar results. After eight weeks of treadmill exercise training and 90 minutes of ischemia followed by 120 minutes of reperfusion, the

pulmonary arteries of sedentary age-matched rats and exercise-trained rats had similar vasodilatory responses to increasing doses of acetylcholine, histamine, and U-46619, despite increased levels of plasma nitrite/nitrate after IR injury in both trained and untrained groups (83). Compared with sedentary, sham-operated rats that did not undergo IR injury, phenylephrine-induced vasocontraction was blunted at submaximal concentrations in both IR-injured sedentary and exercise-trained arteries. Exercise-trained rats that underwent IR injury did have a greater relaxation response to sodium nitroprusside than IR injured and non-injured sedentary rats, suggesting that exercise training increased the sensitivity of vascular smooth muscle in pulmonary arteries.

ANIMAL STUDIES: EXERCISE TRAINING AND ENDOTHELIAL FUNCTION

Though two animal studies suggest that exercise training did not protect against ischemia-reperfusion (IR) injury in the coronary and pulmonary arteries of healthy rats, many studies have suggested that aerobic exercise training's effect on arterial function may be dependent on the duration and intensity of exercise training, the animal model used, and the artery location. For example, less than two weeks of exercise training did not alter acetylcholine-induced vasodilation in healthy rat aortic rings (84), but four (84), eight (64), ten (68, 402) and 12 weeks of exercise training enhanced acetylcholine-induced vasodilation and the training effect was abolished with L-NAME administration (64, 84, 85). In addition, eight weeks of exercise training increased acetylcholine-induced vasodilation in rabbit aortas (63). In other conduit arteries, short-term exercise training (<10 days) increased or was associated with higher endothelial-dependent vasodilation in the porcine coronary (220), pulmonary (184), and brachial (257) arteries as well as canine coronary arteries (321, 378). Similar changes were seen with long-term exercise training (>10 weeks) in rat carotid (199), rabbit pulmonary (63), porcine coronary (219, 268), and porcine female brachial arteries (221). Improvements in vascular endothelial function were attributed to changes within the endothelium itself, perhaps by nitric oxide, because exercise training was associated with greater endothelial-dependent vasodilation and higher levels of eNOS mRNA and protein content in many arteries (84, 184, 219, 385, 402). In contrast, a few studies have suggested no change in endothelial function with long-term exercise training in rabbit carotid (63), porcine pulmonary (182), femoral (221, 256), brachial (256), or coronary (284) arteries. In resistance arteries and arterioles of healthy animals, exercise training of less than four weeks increased (203, 344, 346) or caused no change (220, 344) in endothelial-dependent

vasodilation while exercise training of greater than eight weeks causes increased (215, 223, 255, 268) or no change (179, 215, 223) in endothelial-dependent vasodilation (178). In summary, short-term exercise training in healthy animals appears to improve endothelial function in most arteries, but the effects on endothelial function with long-term aerobic exercise training appear to be specific to the location of the artery (resistance versus conduit) (140, 178).

A clearer picture emerges with aging and disease. Exercise training preserves endothelial function or attenuates endothelial dysfunction in most animal models of aging and cardiovascular disease (178). Ten or more weeks of exercise training attenuated the age-related endothelial dysfunction in rat gracilis (345), soleus (332), and gastrocnemius (333) arterioles and was associated with NOS (332, 333) signaling pathways. In contrast, two studies found that older rats who trained three months had lower acetylcholine-induced aortic vasodilation compared with sedentary controls (156, 371). Yet in animal models of high cholesterol, endothelial function was improved with two to 20 weeks of exercise training in the porcine brachial (386, 388), rabbit (400, 401) and mouse (277) aorta, mouse carotid (300), rabbit femoral (180, 181), and porcine coronary (366, 387) arteries. Similarly, endothelial dysfunction induced by high blood pressure was alleviated after six or more weeks of exercise training in the rat aorta (61, 62, 137), carotid (12, 62), and mesenteric (12) arteries. In heart failure animal models induced by progressive ischemia, four to 16 weeks of exercise training ameliorated the drop in endothelial function in porcine coronary (112, 141, 142) and pulmonary (183), canine coronary (403), and rat carotid (174) arteries. Improvements in endothelial function with training were often accompanied with higher levels of eNOS protein (137, 174, 300, 332) and mRNA content (332, 334) or were abolished by NOS blockade (12, 112), indicating a link between exercise training and nitric oxide.

HUMAN STUDIES: EXERCISE AND ENDOTHELIAL FUNCTION

Consistent with the animal models described above, human studies suggest that aerobic exercise training reverses or alleviates endothelial dysfunction caused by aging and/or cardiovascular disease (140). In healthy humans, endothelial function does not appear to be enhanced by localized exercise such as forearm handgrip training (16, 115, 139) while whole body aerobic exercise training may (72, 87, 135, 161) enhance endothelial function as measured by flow-mediated dilation (FMD) (72) and acetylcholine-induced vasodilation (87, 135, 161). Indeed, one cross-sectional and two interventional studies suggest that chronic aerobic exercise increases nitric oxide bioavailability in older adults. Three months of chronic aerobic exercise increased nitric oxide bioavailability in older women (245) and in middle-aged and older adults (343). In addition, the blockade of nitric oxide synthase caused greater decreases in forearm blood flow in endurance-trained older adults compared with their age-matched sedentary counterparts (349). In the most recent study, basal leg blood flow was unaffected by aerobic exercise training in middle-aged and older adults, which may be due to the increases in sympathetic nervous system activity being offset by increases in nitric oxide bioavailability (343). Indeed, reduced nitric oxide bioavailability, possibly due to increased oxidative stress, is thought to be the primary factor causing impaired endothelial function with aging in humans (38, 105, 176, 327, 350).

In patients with heart failure, four or more weeks of handgrip exercise training in the forearm increased FMD (168) and acetylcholine-induced vasodilation (148). Similarly, four weeks of cycling training increased acetylcholine-induced vasodilation and FMD in the forearms of patients with heart failure (234). Six months of cycling training increased the blood flow response to acetylcholine and basal nitric oxide

formation in the femoral arteries of heart failure patients (147) while in patients with hypertension, 12 weeks of walking increased forearm blood flow responses to acetylcholine and increased the maximal reactive hyperemia response (161, 163). Also, in patients with coronary artery disease, four weeks (146, 149), 10 weeks (130), and five months (126) of cycling (126, 146, 149), walking (130), or rowing (146) increased coronary blood flow in response to acetylcholine (126, 146, 149) and significantly increased posterior tibialis artery FMD (130). Brachial artery FMD was increased after cycling and walking training for 10 weeks, but the increase did not reach significance (130).

The effects of resistance exercise training on endothelial function are harder to interpret because many studies involved both aerobic and resistance exercise combined (152, 246-248, 376, 377), isolated handgrip exercise (16, 168, 195), or diseased populations (374). Only two studies have examined the effects of resistance exercise training on endothelial function in young adults. Thirteen weeks of whole body resistance training did not alter the brachial FMD of young healthy men (302) though 6 weeks of resistance training did increase forearm blood flow in healthy young men (157). As for the interaction of aging with resistance exercise training, the fall in vascular function with aging may be ameliorated as middle-aged resistance-trained subjects had lower vascular resistance than sedentary age-matched controls (262) and middle-aged and older subjects who performed 13 weeks of resistance exercise training had increases in basal leg blood flow and lower femoral vascular resistance (8). In contrast with these findings, a cross-sectional study found that middle-aged men who were resistance-trained had similar responses in carotid vasoreactivity to a cold pressor test as sedentary age-matched controls (196) and an 18-week resistance training intervention study with older post-menopausal women found no changes in brachial FMD (54).

In summary, these studies in humans suggest that endothelial function is enhanced and/or endothelial dysfunction is attenuated or preserved with aerobic exercise training though the effects of resistance training are still unclear. Whether the positive exercise-induced changes in endothelial function seen in older individuals and patients with cardiovascular diseases extends to IR injury remains to be determined. In the next section, the human limb model of vascular IR injury will be discussed along with the possible mechanisms by which aerobic exercise training may alleviate endothelial dysfunction or enhance endothelial function.

HUMAN STUDIES: VASCULAR ISCHEMIA-REPERFUSION INJURY

In vivo studies of the human limbs have shown that 15 to 20 minutes of ischemia and 15 minutes of reperfusion in the forearm impairs endothelial function as measured by brachial artery flow-mediated dilation (FMD) (241, 242, 381), radial artery FMD (133, 201, 236), and acetylcholine-induced increases in blood flow (26, 42, 200, 254, 290, 292, 322). Within an hour, FMD returns to baseline values (201) while endothelial-independent vasodilation appears to be unaffected by IR injury at any time point (42, 201, 241, 254, 292), suggesting that this type of IR injury induces damage to endothelial cells, not vascular smooth muscle cells. Because FMD measured at the brachial or radial artery after five minutes of cuff occlusion is considered a measurement of endothelial-dependent (i.e. nitric oxide-dependent) vasodilation (138), the IR injury described above is considered to cause temporary endothelial dysfunction. Indeed, during ischemia, energy depletion and ROS production occurs when inadequate blood flow to tissues initiates a pathway leading to calcium overload and free radical production by causing intracellular oxygen levels to fall, which inhibits the mitochondria, thereby lowering ATP levels, and consequently, increasing the rate of glycolysis. Based on these changes in the relative mitochondrial and glycolytic activities, lactate increases, hydrogen ions increase, and the pH within the cell is lowered. This fall in pH inhibits nitric oxide production by eNOS. In an attempt to remove hydrogen ions from the cell, the sodium-hydrogen exchanger transports hydrogen ions out while bringing sodium ions into the cell (49). The sodium-calcium exchanger then responds to the increases in sodium ions by moving sodium ions out and calcium ions into the cell (49). This cascade of events leads to increases in intracellular calcium levels and during this cascade, low oxygen levels and

decreased ATP production prime the mitochondria for the production of reactive oxygen species when oxygen levels rise during reperfusion (49).

While the best treatment for ischemia is immediate reperfusion of the tissue with blood, re-entry of the blood into the injured tissues also causes damage through calcium loading, inflammation, and a surge of free radical production (18, 29). With reperfusion, oxygen levels drastically rise with a simultaneous increase in pH levels, leading to a burst of reactive oxygen species formation. For example, superoxide is produced primarily at Complex I (NADH-Ubiquinone Reductase) and III (Ubiquinone-Cytochrome C Reductase) of the mitochondria, superoxide combines with nitric oxide at the endothelium to form peroxynitrite, superoxide dismutase forms hydrogen peroxide from superoxide and hydrogen ions, and on occasion, superoxide forms hydroxyl radicals by the Fenton and Haber-Weiss reactions. The role of oxidative stress in vascular IR injury is supported by studies of young healthy men (292) and women (254, 292), who had blunted acetylcholine-induced increases in forearm blood flow after 20 minutes of ischemia and 15 to 60 minutes of reperfusion. Plasma total antioxidant status fell 15 minutes after reperfusion (254). Similarly, intra-arterial infusion of 60 mg/mL vitamin C during reperfusion blocked the fall in arterial vasoreactivity to acetylcholine and reduced the neutrophil oxidative burst compared with saline infusion (292). An additional set of studies in the same subjects found that the infusion of the nitric oxide synthase inhibitor N-monomethyl-L-arginine (L-NMMA) with and without vitamin C infusion caused forearm blood flow to fall to a similar extent before IR injury, but 15 minutes after IR injury, L-NMMA infusion without vitamin C had less vasoconstriction compared with L-NMMA infusion with vitamin C, suggesting that vitamin C does not enhance protection against ischemia, rather it protects during reperfusion, perhaps by increasing NO bioavailability (292).

Pharmacological studies in the human limb have highlighted the importance of nitric oxide in vascular IR injury. For example, when 5 μg/mL bradykinin was infused into the brachial artery prior to 20 minutes of ischemia and 15 minutes of reperfusion, radial artery FMD was not significantly different from baseline, but radial artery FMD was significantly decreased when saline was infused prior to IR injury (236). Though no changes were found in plasma von Willebrand factor (a marker of endothelial cell damage), plasma nitrate levels dropped significantly 15 minutes after IR injury, but were maintained when bradykinin was infused. This suggests that bradykinin preserved the nitric oxide pathway during vascular IR injury since bradykinin stimulates phosphatidylinositol 3-kinase protein kinase C/Akt, which stimulates NOS to produce nitric oxide (236). This in turn, maintained endothelial function despite the ischemic insult. Similarly, another study using sildenafil citrate found that 15 minutes of ischemia and 15 minutes of reperfusion of the lower arm in 10 healthy men caused radial artery FMD to fall by 6.7%, but was preserved with sildenafil administration (133). Sildenafil primarily inhibits the PDE5 enzyme, which hydrolyzes cGMP in smooth muscle cells. When the breakdown of cGMP is blocked by sildenafil, its concentration rises within the cells and leads to vasodilation (114). PDE5 is found in animal and human hearts (240, 355, 375), animal arterial endothelial cells (194), and has been shown to affect cardiac function (33, 114, 320, 354, 355). This study suggests that increased levels of cGMP, an important step in vasodilation, protect against IR injury in the endothelium of humans.

Damage to surrounding tissues from IR injury itself and increases in oxidative stress from IR stimulate inflammatory pathways, attracting macrophages that release cytokines and direct neutrophils, eosinophils, and other phagocytes to the location of damaged tissues. By engulfing damaged tissue and foreign invaders, this immune system function can increase the formation of reactive oxygen species, which contribute to IR

injury. For example, NADPH oxidase, an enzyme found in neutrophils, mediates the formation of superoxide, which interacts with myeloperoxidase (another enzyme in phagocytes) to form hypochlorous acid, a potent chemical for protein degradation. Superoxide can also combine with nitric oxide, which is found in inflammatory and endothelial cells to produce nitryl chloride or similar derivatives. In addition, another enzyme found in endothelial and inflammatory cells, xanthine oxidase, converts hypoxanthine (formed by mitochondrial respiration during ischemia) into xanthine and superoxide. Xanthine can then be converted by xanthine oxidase again into another superoxide radical and uric acid. The production of these reactive oxygen species and their interaction with calcium during inflammation aids in the removal of infarcted tissues, but may also impair endothelial function due to its effects on nitric oxide. Specifically, studies in the human limbs have shown that the blunted endothelial function after 20 minutes of ischemia and 15 to 30 minutes of reperfusion is associated with increased systemic neutrophil activation as measured by CD11b expression (201), increased platelet-neutrophil complexes as measured by the percentage of neutrophils staining for CD42b (201), and increased oxidative burst by neutrophils as measured by the binding index of spontaneously activated neutrophils (292) in the blood compared with baseline values.

POTENTIAL MECHANISMS OF EXERCISE-INDUCED PROTECTION FROM VASCULAR ISCHEMIA-REPERFUSION INJURY

As mentioned previously, though the exact mechanisms behind the endothelial dysfunction following ischemia-reperfusion (IR) injury are unknown, changes in nitric oxide bioavailability, oxidative stress, and inflammation likely play a role. In the next section, these regulators will be identified and their role in IR injury and vascular adaptations to exercise will be discussed.

Endothelial Nitric Oxide Synthase

Within the coronary arteries and systemic arterial circulation, vascular tone is regulated, in part, by the endothelium via endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and surrounding vascular smooth muscles. In response to unidirectional laminar shear stress, the endothelium produces nitric oxide by two different pathways: through a calcium-independent direct activation of the eNOS enzyme and by calcium-dependent pathway (155). With the chaperone heat shock protein 90 (HSP90) holding eNOS in an active confirmation, increased concentrations of calcium phosphorylate ser1177, which increases the specific activity of eNOS while dephosphorylation of thr495 regulates the association of calmodulin with eNOS, which initiates nitric oxide production (65). In the presence of oxygen, co-factors tetrahydrobiopterin (BH4), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN), and electron donor nicotinamide adenine dinucleotide phosphate (NADPH), nitric oxide is produced in a two-step reaction when eNOS oxidizes the amino acid L-arginine to citrulline and nitric oxide (51, 342). The newly-produced unstable gaseous substance induces vasodilation of nearby blood vessels (less than one millimeter

beyond its site of synthesis) (274) through guanylate cyclase and cGMP or by directly activating calcium-sensitive potassium channels on vascular smooth muscle cells (30).

Improvements in endothelial function with exercise training in animals (137, 174, 216, 217, 219, 222, 268, 300, 332, 334, 387) are associated with increases in eNOS protein expression and activity, as discussed previously. In humans, left internal mammary arteries recovered during surgery in coronary artery disease patients who underwent four weeks of exercise training had higher levels of eNOS protein and mRNA expression, increased phosphorylation of eNOS, and greater endothelial-dependent vasodilation than sedentary patients (146). Because aerobic exercise training appears to upregulate eNOS expression and activity, this may increase nitric oxide production, providing protection against vascular IR injury.

Endothelial Nitric Oxide Synthase Cofactors and Substrates

When L-arginine or tetrahydrobiopterin (BH4) levels are low, eNOS becomes uncoupled, producing superoxide instead of nitric oxide (51), even in the presence of L-NAME (340). This has been demonstrated in vitro (373, 390), in coronary arteries of dogs (78), and in healthy (166) and diabetic (52) human aortic endothelial cells. Not only does this decrease nitric oxide production, it also increases nitric oxide breakdown because superoxide generated by eNOS or other sources can combine with existing nitric oxide to produce peroxynitrite, a reactive oxygen species. Both of these processes lower nitric oxide bioavailability, contributing to endothelial dysfunction. Several studies in humans have highlighted the importance of BH4 in peripheral vascular endothelial function. Infusion of BH4 improved acetylcholine-induced vasodilation in type II diabetics (160) and chronic cigarette smokers (159), but was attenuated when a NOS inhibitor (L-NMMA) was co-infused. In healthy men ages 19 to 81 years old (162) and hypercholesterolemic subjects (341), serotonin-induced vasodilation was restored after

the infusion of BH4. Another study using saphenous veins and internal mammary arteries removed from patients undergoing coronary artery bypass grafting found that higher vascular levels of BH4 were positively associated with acetylcholine-induced vasodilation, eNOS coupling, and lower superoxide production (9).

A similar trend has also been seen with vascular IR injury. Twenty minutes of ischemia and 15 minutes of reperfusion in the human forearm impaired acetylcholine-induced increases in forearm blood flow and decreased plasma total antioxidant status 15 minutes and four hours after cuff release (254). When R-BH4, S-BH4 (an antioxidant stereoisomer with a weak affinity for eNOS), and NH4 (structurally-similar chemical with antioxidant properties, but no interaction with eNOS) were administered, the impaired acetylcholine-induced vasodilation previously seen with IR injury disappeared, suggesting that BH4 may play a more important role in free radical scavenging than protection of eNOS coupling (254). Indeed, invasive studies of myocardial IR injury in rats and porcine coronary arteries have shown a protective effect of BH4 administration (368, 396-398) and a loss of protection when BH4 production was inhibited by 2,4-diamino-6-hydroxypyrimidine (395, 397).

Based on the findings that BH4 is crucial for nitric oxide production and lowering superoxide production, does aerobic exercise training increase BH4 bioavailability? When BH4 was orally supplemented in older sedentary men, brachial flow-mediated dilation (FMD) was higher than placebo, matching that of young sedentary and older trained men (106). Moreover, a study in rat aortas suggests that BH4 biosynthesis is increased by shear stress from increased blood flow (211). Eight weeks of exposure to increased blood flow without changes in mean arterial pressure from aortocaval fistulas enhanced the expression of eNOS, caveolin-1, phosphorylated Akt, phosphorylated eNOS Ser1177, and the rate limiting enzyme for BH4 synthesis, GTPCH I. cGMP and

BH4 levels were augmented also, suggesting that blood flow does not affect the oxidation of BH4, but does stimulate BH4 synthesis through increased expression and activity of the rate limiting enzyme GTPCH I (211). Because exercise increases shear stress on the endothelium through increases in blood flow (360), it is possible that exercise may increase BH4 levels by altering the enzyme that produces BH4. Taken together, these results suggest that aerobic exercise training may positively affect BH4 levels, which may lead to protection against IR injury.

L-Arginine bioavailability to eNOS is a balance between its breakdown by arginase and production primarily in the kidneys and intestines (67). After the kidneys convert citrulline found in the blood into L-arginine, it must be transported into the endothelial cell by cationic amino acid (CAT-1) transporters because intracellular stores of L-arginine are unavailable to eNOS ("the L-arginine paradox") (67). As mentioned previously, when L-arginine levels are insufficient, eNOS produces superoxide instead of nitric oxide (373), causing not only increases in oxidative stress, but also decreases in eNOS-produced nitric oxide. While L-arginine supplementation may (173, 287) or may not (79, 98) increase endothelial-dependent vasodilation in young, healthy humans, the role of L-arginine in ameliorating endothelial dysfunction induced by cardiovascular disease is supported by human and animal studies. Supplementation with L-arginine improved endothelial function as measured by acetylcholine-induced dilation or FMD in patients with hypercholesterolemia (71, 74), coronary artery disease (3), hypertension (351), and heart failure (165, 207, 296, 303). Cardiovascular disease-related endothelial dysfunction may be due to increased levels of arginase, decreased production of L-arginine, or alterations in the CAT-1 transporter (197, 316), but age-associated declines in endothelial function do not appear to be associated with alterations in L-arginine transport in the human forearm (5). Whether L-arginine supplementation improves the

endothelial dysfunction seen with ageing is unclear. Two weeks of L-arginine supplementation significantly increased brachial FMD in humans over 70 years old (25) while acute intravenous infusion of L-arginine did not alter brachial FMD in healthy older adults (121) or post-menopausal women (24). L-arginine infusion did not enhance the forearm blood flow responses to any dose of acetylcholine in normotensive subjects less than 30 years old, but at higher doses of acetylcholine, forearm blood flow responses were significantly elevated with L-arginine infusion in subjects ages 31 to 73 (351). It should be noted that cigarette smokers were included in the study, but were evenly distributed throughout the different age groups. Similarly, aging was associated with lower acetylcholine-induced peak coronary blood flow in patients with atypical chest pain, but L-arginine infusion normalized the blunted coronary blood flow (60).

In terms of its effects on ischemia-reperfusion (IR) injury, six weeks of L-arginine supplementation improved the coronary artery response to serotonin after myocardial IR injury in young and adult male rats, but not in infant or elderly rats (271). In porcine hearts exposed to 30 minutes of ischemia and 90 minutes of reperfusion, nitric oxide production was inhibited and arginase activity was increased (158). Immunohistochemical measures in arterioles showed an upregulation in arginase expression and downregulation of NOS expression. Functionally, arterioles that underwent IR injury had less dilation in response to adenosine and serotonin compared with arterioles from non-ischemic regions of the heart. The blunted dilation response was restored when arginase was inhibited by α-difluoromethylornithine or with L-arginine treatment. Like the porcine model, IR injury lowered acetylcholine-induced relaxation of feline coronary artery rings, but vasorelaxation was preserved with L-arginine treatment (382). IR-stimulated release of TNF-α may upregulate arginase within endothelial cells (119), leading to increasing superoxide production. As for exercise training, shear stress

increases L-arginine uptake in porcine aortic endothelial cells (294), but only one study has examined the effects of exercise training on L-arginine transport in humans. Patients with coronary heart failure who exercise trained for eight weeks had higher levels of arginine transport than untrained patients and this was associated with higher acetylcholine-induced vasodilation in the forearm (289). If exercise training enhances L-arginine production, transport, and uptake in healthy humans is unknown.

More recently, two other regulators of eNOS activity have been identified. Heat shock protein 90 (HSP90) associates with eNOS at amino acid sequence 310-323 (391), holds eNOS in a protein confirmation that favors the phosphorylation of ser1177 (91, 120), activating the protein kinase B/Akt pathway and dephosphorylation of thr495 by calcineurin (209), leading to activation of eNOS (76, 91, 118, 342) despite drops in calcium concentration (43). Binding of caveolin-1 to eNOS, on the other hand, keeps eNOS in a primed, yet inactive state (136). Experiments using isolated endothelial cells have shown that shear stress increases eNOS expression (21, 269, 278) and eNOS-HSP90 interaction (120). Disruption of this interaction increases superoxide production and decreases nitric oxide production (120, 297, 342) and HSP90 may also decrease superoxide production independently of its effects on nitric oxide production (342).

HSP90 appears to be cardioprotective against IR injury in several animal and isolated cell models. Overexpression of HSP90 in the porcine vasculature reduced myocardial dysfunction and infarct size, which was abolished with L-NAME administration (209). Similarly, when HSP90 was inhibited by geldanamycin, myocardial functional recovery from IR injury in neonatal rabbit hearts was blunted (324). A strain of rats that are especially resistant to myocardial IR injury were found to have higher levels of nitric oxide production and HSP90-eNOS interactions. Administration of geldanamycin lowered the resistance of these hearts to ischemia (325).

Despite the apparent importance of HSP90 and caveolin-1 in regulating eNOS activity, the effects of exercise training on their levels are inconclusive. Endurance-trained runners had similar baseline levels of HSP90 in leukocytes as untrained individuals and an acute bout of exercise did not cause changes in HSP90 levels in either group (323). After a half-marathon, no changes were seen in the levels of HSP90 in leukocytes (111) though exercise training for ten days in the heat increased levels of HSP90 in human leukocytes (258). In male rats, one bout of exercise increased the synthesis of a 90 kD protein believed to be HSP90 in the skeletal muscle and spleen (238). In terms of exercise-induced protection against myocardial IR injury in animals, three to five days of treadmill running by female rats resulted in greater myocardial function after IR injury than non-exercise controls (150). HSP90 levels were significantly higher in rats trained in a warm environment than controls and though it did not reach significance; rats trained in a cold environment also had higher levels of HSP90 than controls. Elevated levels of HSP90 were also seen in male rats exercising three and six weeks in the cold, but not in ambient temperatures and the elevated levels were no longer significant after nine weeks of exercise in the cold (154). As for caveolin-1, 16-20 weeks of aerobic exercise training did not alter caveolin-1 levels in coronary (366) or brachial (386) arteries of pigs. In summary, alterations in HSP90 and caveloin-1 levels with exercise training are not likely and therefore are not expected to participate in exercise-induced vascular protection against IR injury. Of the factors regulating eNOS, the current literature suggests that the upregulation of eNOS activity and expression by aerobic exercise training is the most promising.

Oxidative Stress

Studies of exercise-induced cardioprotection in animal hearts suggest a possible role of exercise in altering the heightened levels of oxidative stress associated with

ischemia-reperfusion (IR) injury. If aerobic exercise training can lower oxidative stress, whether by increasing antioxidant levels, decreasing free radical formation, or a combination of both, the breakdown of nitric oxide may be reduced and initial injury to tissues may be lessened, lowering oxidative stress and inflammation; all of which help to maintain endothelial function. In the same context, if exercise training can increase nitric oxide bioavailability, it may lower oxidative stress and inflammation by nitric oxide's direct interaction with the mitochondria, other producers of reactive oxygen species, and inflammatory cytokines. During ischemia, mitochondria are the greatest source of oxidant production (49). As mitochondrial respiration increases, electron flux through the electron transport chain increases, raising the risk of electron loss, especially from Complex I (NADH-Ubiquinone Reductase) and Complex III (Ubiquinone-Cytochrome C Reductase) (34, 35, 164, 239, 372). Escaped electrons combine with oxygen to produce superoxide which left unquenched, can damage surrounding tissues in its search for another electron. A recent animal study suggests that after exercise training, free radical generation in the mitochondria is decreased (335). Male rats that trained 16 weeks had lower hydrogen peroxide production at Complex I in the myocardial mitochondria compared with sedentary age-matched controls, which was blocked by rotenone administration (a chemical that inhibits electron flow from succinate to Complex I). Another study in rat hearts has also shown that the binding of nitric oxide to Complex I of the mitochondria protects against IR injury (270) and binding of nitric oxide to cytochrome C oxidase may also provide protection against IR injury. When oxygen concentrations are high in the cell and electron turnover is low, nitric oxide interacts with cytochrome C oxidase, increasing the reduced fraction of cytochrome cc1, elevating the reductive pressure and increasing electron turnover on the portion of the cytochrome C oxidase that is not bound, which helps maintain steady state respiration (103). At low

concentrations of oxygen, such as that seen during ischemia, when electron turnover within the mitochondria is high, "the high affinity interaction of nitric oxide with reduced species of the catalytic cycle will result in inhibition of respiration" (103). In this way, nitric oxide acts to maintain homeostasis in the face of falling oxygen concentrations (48, 318). In addition, nitric oxide has been shown to contribute to adenine nucleotide monophosphate kinase (AMPK) activation in animal hearts (231) and isolated cell preparations (70), which stimulates glycolysis during ischemic conditions in animal hearts (312, 404) and skeletal muscle (265).

Along with the mitochondria, eNOS uncoupling, xanthine oxidoreductase, and NADPH oxidase may contribute to the increases in oxidative stress with reperfusion (266). NOX2, a type of NADPH oxidase located in the plasma membrane of human endothelial cells (19, 39) and vascular smooth muscle cells (370), mediates reactive oxygen species production and may be especially important in lowering nitric oxide bioavailability (134). Indeed, the age-associated decline in endothelial function in the conduit arteries of humans is associated with an increased expression of NADPH oxidase p47phox in endothelial cells (94) and obese and overweight adults also have higher endothelial cell p47phox expression (326). On the other hand, nitric oxide may downregulate NOX1 (293), a type of NADPH oxidase in vascular smooth muscle cells (39), but nitric oxide's effects on NOX2 have yet to be elucidated. Aerobic exercise training may lower the expression of NADPH oxidase in humans with cardiovascular disease (4, 288). After four weeks of exercise training, left internal mammary arteries of coronary artery disease patients had lower gp91phox, p22phox, and NOX4 protein expression and activity, which was accompanied by lower reactive oxygen species generation and higher acetylcholine-mediated vasodilation than non-exercising patients (4) and six months of aerobic exercise training in individuals with numerous risk factors

for cardiovascular disease decreased systemic measures of oxidative stress and altered p22phox expression compared with pre-exercise values (288). Whether aerobic exercise training alters NADPH oxidase expression in healthy adults has yet to be determined.

Xanthine oxidoreductase's role in IR injury has been complicated by species differences in the tissue distribution of this enzyme (283). Some studies suggest that there is little xanthine oxidoreductase activity in human hearts or leukocytes while rats have much higher levels (185, 204, 380). Human studies in the arteries of heart failure patients (92, 109, 123) and diabetics with hypertension (50) suggest a presence of xanthine oxidoreductase as the xanthine oxidase inhibitor, allopurinol, increased acetylcholine-induced blood flow in the forearm as compared with placebo. With advancing age; however, the blunted endothelial dependent vasodilation does not appear to be related to xanthine oxidase expression or activity. Brachial FMD was not improved with allopurinol administration in older subjects (104) and endothelial cells from young and older healthy adults had similar levels of xanthine oxidase protein expression (94, 104) As for exercise training, studies in humans and animals are almost exclusively devoted to skeletal muscle, exhaustive exercise bouts, or acute bouts of exercise. Though perhaps not applicable to humans, one study in rat aortas found that after eight weeks of aerobic exercise training, xanthine oxidase activity was no different than sedentary controls (170). Surprisingly, other studies suggest a protective role of xanthine oxidoreductase in IR injury. In environments of low oxygen concentrations, xanthine oxidase can produce nitric oxide by nitrite or nitrate reduction (128, 229, 230, 409) . Specifically, in rat hearts exposed to 30 minutes of ischemia, nitrite administration lowered infarct size and was associated with higher xanthine dehydrogenase and xanthine oxidase activity and nitrite-induced cardioprotection was abolished when flavoprotein reductases and the molybdenum site of xanthine oxidoreductase were inhibited (15).

Superoxide produced by these enzymes has several possible fates, depending on the surrounding environment. In the presence of nitric oxide, superoxide is converted to peroxynitrite, a reactive nitrogen species that can lower nitric oxide bioavailability directly or indirectly. If superoxide dismutase is nearby, it will convert superoxide into hydrogen peroxide and water. Two main types of superoxide dismutase are found in the cell, manganese superoxide dismutase (MnSOD) in the mitochondria and copper-zinc superoxide dismutase (CuZnSOD) in the cytosol. Outside of the cell is extracellular copper-zinc superoxide dismutase (EcSOD). In the myocardium of rats, short-term (86, 116, 150, 227, 228, 399) and long term (172, 191, 232, 250, 328, 331) exercise training was associated with higher MnSOD activity or protein expression, yet other studies report lower activity with long-term training (264) or no change in activity or protein content with short (46, 226, 361) and long-term exercise training (154, 367). Additionally, with short-term exercise training in a cold environment, MnSOD activity was decreased (361) or increased with three weeks and six weeks of exercise training (154). Unlike MnSOD, most studies suggest that CuZnSOD in the myocardium is unaltered with exercise training in rats. Six short-term exercise training studies found no change in CuZnSOD levels (86, 116, 150, 226, 227, 301) and only three studies found its activity or protein expression to be increased (154, 172, 330). After three and six weeks of treadmill exercise training in rats, CuZnSOD activity was higher than controls, but this elevation was not significant after nine weeks of exercise (154). Also, rats that underwent ten weeks of endurance exercise training had higher CuZnSOD activity within the heart than sedentary controls (330).

Within the vasculature, more than eight weeks of exercise training produced higher protein levels and/or activity of CuZnSOD in porcine aortas (311), coronary arterioles (310), and femoral arteries (222) as compared with sedentary controls. In the

rodent animal model, MnSOD (170, 171) and CuZnSOD (171) activity and protein expression were increased in rat aortas and CuZnSOD protein content was higher in the popliteal arteries and arteries feeding the white gastrocnemius skeletal muscle of exercise-trained rats (223) compared with their sedentary counterparts. Human aortic endothelial cells also had increased CuZnSOD expression with increased shear stress (175). Though human vascular studies are lacking, most mammalian species have similar levels of MnSOD and CuZnSOD in their arteries and so these results may apply to humans as well (339). EcSOD protein levels were also higher in the aortas of mice that trained three weeks on a treadmill (82, 117). Of the studies investigating the relation of exercise and arterial antioxidant content and activity, special emphasis is placed on EcSOD because it is produced in smooth muscle cells (339) and is secreted into the extracellular space adjacent to the endothelium. This superoxide dismutase enzyme is thought to play an important role in nitric oxide bioavailability (108, 286, 383) and because rats have extremely low levels of EcSOD in their arteries (251), exercise studies using pigs, cows, and mice, which have similar arterial levels of EcSOD as humans (339), are especially relevant. Indeed, human studies of cardiovascular disease suggest an important role of EcSOD in maintaining endothelial function. Patients with coronary artery disease (213) and coronary heart failure (214) have reduced EcSOD activity, which is negatively correlated with measures of radial artery FMD. Overall, studies in humans and animals suggest that exercise training is linked with higher levels of MnSOD and EcSOD within the heart and all three superoxide dismutase isoforms may be elevated with exercise training in the vasculature. Oxidative stress will be estimated by whole blood GSH:GSSG levels and plasma F2-isoprostanes.

Superoxide dismutase converts superoxide into hydrogen peroxide, a freely diffusible, but less potent reactive oxygen species. On its own, hydrogen peroxide is not

very damaging to endothelial cells and may act as a second messenger, but in the presence of myeloperoxidase, can react to produce the more damaging hypochlorous acid (408) or peroxynitrite (210). Working together, the antioxidant enzymes catalase and glutathione peroxidase quench hydrogen peroxide within the myocardium and vasculature. Catalase has a high Km for hydrogen peroxide, acting when concentrations of hydrogen peroxide are very high while glutathione peroxidase works to lower oxidative stress when hydrogen peroxide concentrations are moderate (153, 347). After three days of exercise training on the treadmill, the left ventricles of rats had increased catalase activity compared with sham controls one day (116, 154, 226-228) and three days after their last bout of exercise (226). However, this rise may be temporary since two studies initially found higher levels of catalase, but these dropped to levels equal to sedentary controls nine days after their last bout of exercise (226) and after six weeks of exercise training (154). Even though some short-term exercise training studies found no change in catalase activity with exercise (86, 150, 361), rats that underwent 8-10 weeks of exercise training had higher catalase mRNA (331) and activity (172, 330, 331) in heart tissue than sedentary controls.

As for the role of exercise in altering the glutathione redox system within the myocardium, rats that exercised three days in a cold environment had higher levels of glutathione peroxidase (150) and rats exercised in a thermoneutral environment had higher levels of reduced glutathione (GSH) (86) than sedentary controls. After eight weeks of aerobic exercise training, trained rats had higher levels of GSH, GSH:GSSG ratio, and glutathione peroxidase than sedentary controls (172). Moreover, older rats that exercised nine weeks had higher glutathione peroxidase mRNA and activity than sedentary controls (331) and after ten weeks of treadmill exercise training, rats had higher glutathione peroxidase activity in myocardial mitochondria and an increased GSH:GSSG

ratio within the cytosolic fraction of the heart (330). Other studies in rat hearts found no change or lower glutathione peroxidase (86, 154, 226, 301, 335), glutathione reductase (86) and GSH (228) expression in exercise-trained rats (190) compared with controls.

Fewer studies have examined the effects of exercise training on catalase and glutathione peroxidase within the vasculature. Sixteen weeks (311) and six weeks (137) of exercise training did not alter catalase protein levels in porcine (311) or rat (137) aortas although two studies found that exercise-trained rat aortas had higher levels of catalase activity and protein expression than controls after eight weeks (170, 171). These same studies found higher activity levels and protein expressions of glutathione peroxidase, GSH, and glutathione reductase after eight weeks of exercise training in rat aortas (170, 171). No other animal studies of exercise and blood vessel concentrations of the glutathione redox systems are known; however, studies of cultured endothelial cells found increased glutathione peroxidase-1 mRNA expression and activity (353) and increased intracellular GSH levels (267) in response to unidirectional shear stress. In addition, intervention studies in humans using aerobic exercise training have shown an increase in basal GSH levels (99), glutathione peroxidase activity (100, 110), and increased glutathione reductase (100) within the blood compared with baseline values before training and sedentary controls. In one study, four months of detraining after four months of exercise training caused blood glutathione peroxidase activity levels to return to pre-training values, suggesting that exercise training caused the alterations in the antioxidant enzyme (110). In summary, exercise training appears to favorably alter the glutathione redox system, which may be especially important within the vasculature. This provides another mechanism by which aerobic exercise training may protect against vascular IR injury.

Potassium Channels

By increasing nitric oxide and cGMP through protein kinase C (80) and extracellular-signal-regulated kinase pathways (313) that stimulate iNOS and eNOS mRNA and protein expression (81, 314), mitochondrial and sarcolemmal ATP-sensitive potassium (KATP) channels can be directly activated by nitric oxide or indirectly through cGMP and protein kinase G (10, 14, 208, 279, 281, 285, 315, 392). It is hypothesized that the opening of KATP channels during cardiac stress (such as ischemia, exercise, etc) combats the loss of ionic gradients within myocytes (192) by hyperpolarizing and shortening the cardiac action potential, lowering calcium influx and ATP consumption (356), thereby attenuating myocardial IR injury. KATP channel activation may also stimulate Bcl-2, an anti-apoptotic protein, which helps protect the permeability of the mitochondrial membrane directly and indirectly through the inhibition of intracellular calcium loading and reactive oxygen species (208). Indeed, cardioprotection against IR injury by ischemic preconditioning (90, 143), perconditioning (317), and remote preconditioning (205) involves the activation of KATP channels. Animal studies examining the role of exercise-induced cardioprotection from IR injury suggest a greater role of sarcolemmal KATP channels than mitochondrial KATP channels. Rats trained 12 weeks on a treadmill had smaller myocardial infarction size compared with controls and this was unaffected by 5-hydroxydecanoate (5-HD), a mitochondrial KATP channel blocker (44). Similarly, short-term exercise training in dogs reduced infarct size and treatment with 5-HD ameliorated this effect only when exercise was performed immediately before IR injury, but 5-HD did not attenuate the decrease in infarct size when exercise was performed 24 hours before IR injury (93), suggesting a possible role of mitochondrial KATP channels in early phase, but not late-phase, cardioprotection. In contrast, sarcolemmal KATP channels do appear to be involved in the cardioprotective

effects of both short-term (<5 days) and long-term exercise training (>12 weeks) in male (66) and female (44, 66) rats as demonstrated using the sarcolemmal KATP channel blocker, HMR 1098. Moreover, the protein expression of two subunits of sarcolemmal KATP channels, SUR and Kir6.2, both of which are required to form a functional potassium channel (356), appear to increase with exercise training in rat hearts (44, 46).

Like the myocardium, KATP channels are present in vascular smooth muscle cells (40) and endothelium (59, 194). The opening of KATP channels causes membrane hyperpolarization, closing of voltage-dependent calcium channels, and lowering of calcium levels within smooth muscle cells (17). The fall in smooth muscle cell intracellular calcium concentration causes smooth muscle cell relaxation and subsequent increases in arterial diameter. In rat and mouse aortas, the immediate cessation of blood flow caused KATP channel-linked membrane depolarization in the endothelium and production of reactive oxygen species by NADPH oxidase (252). In the same context, shear stress, a cultured-cell model of exercise, increased the expression of KATP channels in rat and bovine vascular endothelial cells (59). As mentioned earlier, ischemia preconditioning in the heart involves KATP channels and the same is true in the peripheral vasculature. Twenty minutes of forearm ischemia caused endothelial dysfunction as measured by acetylcholine-induced increases in forearm blood flow, but ischemic preconditioning of the limb prior to IR lessened the fall in endothelial function (42). Inhibition of KATP channels with glibenclamide abolished the protection by ischemic preconditioning and opening of KATP channels by diazomide prevented endothelial dysfunction caused by forearm ischemia. Similarly, endothelial dysfunction, as measured by radial FMD, was induced by 15 minutes of forearm ischemia, but sildenafil, a drug that increases cGMP levels, attenuated the IR-induced endothelial dysfunction (133). Administration of glibenclamide abolished the protection conferred

by sildenafil, suggesting a role of KATP channels in endothelial dysfunction caused by IR injury. Another study in humans found that remote ischemic preconditioning and remote postconditioning in the limbs attenuated endothelial dysfunction as measured by brachial FMD from 20 minutes of forearm ischemia (243). Blockade of KATP channels abolished the protective effects of pre and postconditioning. In summary, these studies suggest that IR injury in the limb vasculature of humans is mediated by nitric oxide through alterations in KATP channel opening. If exercise training can alter nitric oxide bioavailability or alter KATP channel expression and/or sensitivity, endothelial dysfunction caused by IR injury may be attenuated.

Inflammation

Cellular damage from ischemia-reperfusion (IR) injury itself and oxidative stress stimulate inflammatory pathways in an attempt to repair damaged tissues. Macrophages stimulate the release of the pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which induce endothelial cells to release adhesion molecules and chemokines, leading to the local recruitment of neutrophils and other leukocytes such as polymorphonuclear cells (169, 177). Increased levels of circulating IL-1β and TNF-α then stimulate the production of interleukin-6 (IL-6) and the release of other inflammatory mediators from the liver, including C-reactive protein (CrP), amyloid A, and fibrinogen (177). This has been well documented with myocardial IR injury in several animal models (101, 102, 235, 307, 308) and TNF-α treatment is associated with blunted acetylcholine-induced vasodilation after IR in mouse (407) and porcine (406) coronary arteries and may upregulate arginase within endothelial cells (119). Studies in human limbs have also shown blunted endothelial function after 20 minutes of ischemia and 15 to 30 minutes of reperfusion and this is associated with increased systemic neutrophil activation as measured by CD11b expression (201), increased platelet-

neutrophil complexes as measured by the percentage of neutrophils staining for CD42b (201), and increased oxidative burst by neutrophils as measured by the binding index of spontaneously activated neutrophils (292) in the blood compared with baseline values. Xanthine oxidase activation by TNF-α also contributes to reactive oxygen species production in the vasculature after IR (406, 407) along with NADPH oxidase. If exercise training can attenuate initial IR injury (thereby lowering the inflammation response), lower oxidative stress, or mediate the inflammation response, then endothelial dysfunction may be attenuated after IR injury.

Large-scale epidemiological studies have suggested an association between higher levels of physical activity and lower levels of CrP (1, 11, 69, 113, 122, 212, 304, 379), IL-6 (259, 304), IL-1 (259), TNF-α (259), fibrinogen (1, 379) in several populations. Similarly, intervention studies comparing pre- and post-training values have found decreases in CrP (107, 131, 338), TNF-α (329), IL-1 (131, 329), IL-6 (131), CD11b (291), CD49d (291), vascular cell adhesion molecule-1 (VCAM-1) (2), intercellular adhesion molecule-1 (ICAM-1) (2), TNF-receptor (73, 225) with training while others found no change in CrP (272, 280), IL-6 (272, 338), IL-1 (338), ICAM-1 (272, 369), VCAM-1 (272), or E-selectin (369) in blood effluent. Regardless, these studies are difficult to interpret because they have no control group. Controlled intervention studies are few and most studies suggest that circulating inflammatory markers are not reduced with training (198). After six months of exercise training, healthy older adults had similar serum CrP levels to sedentary controls, despite an increase in maximal oxygen consumption in the endurance-trained group (151). Similarly, 16 weeks of exercise training in overweight, insulin-resistant adults did not reduce CrP levels compared with sedentary controls (249). After eight weeks of exercise training in chronic heart failure patients, circulating TNF-α, TNF-receptor 1, TNF-receptor 2, IL-6, CD14, E-selectin, and

ICAM-1 were no different than age-matched sedentary controls (276). Another study in chronic heart failure patients also found that serum TNF-α, IL-6, and IL-1β levels were similar in patients that exercised six months and untrained controls; however, biopsies found a significantly lower expression of TNF-α, IL-6, and IL-1β in skeletal muscles of trained than controls (125). In contrast, a study in healthy adults found that after nine months of intense training for a marathon, circulating CrP levels significantly decreased pre- to post-training while no change in CrP levels was observed in untrained controls (253). In summary, the relatively few exercise training intervention studies, especially in healthy individuals, make conclusions regarding exercise training's effects on inflammation difficult.

Chapter 3: Endurance Training, Resistance Training, and Vascular Ischemia-Reperfusion Injury

INTRODUCTION

Cardiovascular disease is the leading cause of death in the United States and most industrialized countries, and coronary heart disease accounts for over half of all cardiovascular-related deaths (237, 306, 405). An estimated 16 million Americans have experienced a myocardial infarction, and approximately 2,400 die from cardiovascular disease each day (237). During a heart attack, energy depletion and reactive oxygen species production occur from inadequate blood flow to tissues and with reperfusion, re-entry of the blood into ischemic tissues causes additional damage through calcium loading, inflammation, and a surge of free radical production (167). This damage termed ischemia-reperfusion (IR) injury can also occur during cardiac surgery to treat cardiovascular disease (53). Traditional coronary artery bypass graft surgery requires cardiopulmonary bypass and the introduction of arterial or venous grafts, rendering the heart and blood vessels ischemic for brief periods of time.

Regular exercise, in addition to heat stress, oxidative stress, ischemic preconditioning, stretching, and pharmacotherapy, is currently being investigated for its possible role in preventing and/or attenuating IR injury in the cardiovascular system (362). In animal models, aerobic exercise training protects against myocardial IR injury (13, 144, 145) and attenuates endothelial dysfunction, an independent predictor of future cardiovascular events (41, 58, 193, 260, 263, 275). In humans, habitual exercise has been shown to attenuate endothelial dysfunction with age and alleviate decreases in endothelial function induced by cardiovascular disease, perhaps by increasing nitric oxide bioavailability, lowering oxidative stress, and reducing inflammation (140, 155, 218,

309). It is currently unknown however, if habitual exercise protects against cardiovascular IR injury in humans. This area of research is difficult to investigate because IR injury in the heart cannot be safely induced in healthy humans, and patients who undergo cardiac surgery typically have concomitant diseases (e.g. hypertension, diabetes) that complicate data interpretation. If exercise training can improve nitric oxide bioavailability, lower oxidative stress, and reduce inflammation, thereby maintaining endothelial function after IR injury, it may become an important non-pharmacological strategy for the prevention and treatment of vascular IR injury.

Accordingly, we utilized a human forearm model (201, 241, 242) involving 20 minutes of limb ischemia as a surrogate model for the heart to determine the association between exercise training and vascular IR injury. We hypothesized that after IR injury, the fall in brachial artery flow-mediated dilation (FMD), an index of vascular endothelial function, would be greatest in sedentary adults while IR injury would be attenuated in endurance-trained and resistance-trained adults. Moreover, within 45 minutes after IR injury, brachial artery FMD values would be back to baseline levels in all groups, but endurance-trained and resistance-trained individuals would have a faster recovery to baseline levels than sedentary individuals.

METHODS

SUBJECTS. A total of 33 apparently healthy adults (27 men and six women) aged 18 to 42 years were recruited from The University of Texas at Austin and surrounding community (Table 3.1). All subjects were normotensive (<140/90 mmHg), non-obese (body mass index <30 kg/m^2), non-smoking, free of overt cardiovascular or other chronic diseases, and were not taking any cardiovascular-acting medications as assessed by a self-reported medical history questionnaire. Exercise-training status was verified by a physical activity questionnaire and maximal oxygen consumption (Table 3.2). Sedentary subjects reported engaging in no exercise or <2 hours of exercise per week. Resistance-trained subjects had lifted weights targeting all major muscle groups >2 times per week for >1 year and reported engaging in moderate and/or strenuous resistance exercise two to six hours per week. Endurance-trained subjects reported cycling and/or running >6 hours per week. The Human Research Committee at The University of Texas at Austin reviewed and approved all procedures, and written informed consents were obtained from all subjects.

PROCEDURES. Subjects reported to the laboratory twice; one session for measurements of arterial blood pressure, arterial stiffness, body composition, and maximal oxygen consumption and another session for measures of endothelial function and blood samples. For women, all vascular measures (arterial stiffness and flow-mediated dilation measures) were performed during the early follicular phase of the menstrual cycle to control for the effects of estrogen on endothelial function (57).

TESTING SESSION 1. Prior to the first testing session, all subjects were >4 hours fasted and abstained from caffeine. Body composition was measured by dual energy x-ray absorptiometry (Lunar DPX, General Electric Medical Systems, Fairfield,

Connecticut), and arterial blood pressure and arterial stiffness were measured simultaneously using a validated automatic device (VP-2000, Colin Medical, San Antonio, Texas) (77). Each subject rested supine for at least 10 minutes in a quiet, dimly lit, temperature-controlled laboratory room. Bilateral brachial and ankle arterial blood pressures were measured with the oscillometric pressure sensor method. Carotid and femoral arterial pulse waves were obtained using arterial applanation tonometry incorporating an array of 12 micropiezoresistive transducers. The femoral tonometry sensor was secured in place by a Velcro strap while the carotid sensor was held in place by a plastic collar on the common carotid artery. Aortic pulse wave velocity, a measure of arterial stiffness, and carotid augmentation index, an index of arterial stiffness and wave reflection, were measured. Pulse wave velocity was calculated from the distance (carotid to femoral artery) divided by transit time (the time delay between the carotid and femoral "foot" waveforms) (358). Carotid augmentation index was calculated as the ratio of the amplitude of the pressure wave above its systolic shoulder to the total pulse pressure as previously described (358). Radial and calculated aortic augmentation index were measured using applanation tonometry and software-derived transformation algorithms (SphygmoCor, AtCor Medical, Inc., Lisle, Illinois). Maximal oxygen consumption (VO_2max) was measured during a modified Balke incremental treadmill exercise test (1% grade increase per minute at individualized treadmill speed) as previously described (357). Oxygen consumption (indirect calorimetry via respiratory gas measurements; Physio-Dyne, Quogue, New York), heart rate, and ratings of perceived exertion (the original Borg scale) (32) were measured throughout the protocol.

TESTING SESSION 2. For the 48 to 72 hours prior to the last testing session, subjects followed and recorded a nitrate-free diet adapted from a diet created by the National Heart, Blood, and Lung Institute (273), which did not allow the ingestion of

foods containing nitrates: vegetables or vegetable products, legumes, cured or processed meats, cheese, seafood or fish, alcohol, strawberries, melons, bananas, or potatoes. Adherence to the nitrate-free diet was verified by diet records, which were subsequently analyzed by a registered dietician. Subjects did not consume vitamin, mineral, and herbal supplements >2 weeks prior to the last testing session. Testing sessions were performed in the morning to minimize possible diurnal changes in dependent variables and subjects arrived at least 10 hours fasted and >20 hours post-exercise.

Brachial blood pressure was measured two to three times using the oscillometric pressure sensor method after subjects had rested in the supine position at least 10 minutes in a quiet, dimly lit, temperature-controlled (23 to 26°C) laboratory room. Endothelial function of the brachial artery was assessed by flow-mediated dilation (FMD) in the right arm before and 15, 30, and 45 minutes after 20 minutes of lower-arm cuff occlusion (Figure 3.1) using an ultrasound machine (iE33, Philips Medical Systems, N.A., Bothel, Washington) equipped with a high-resolution linear array transducer as previously described (88). At each timepoint, longitudinal images of baseline brachial artery diameters were recorded proximal to the forearm cuff for an average of 90 seconds prior to forearm occlusion with a rapid cuff inflator (E20 Inflator, AG101 Air Source, and Rapid Version Cuffs, Hokanson, Inc., Bellevue, Washington) set to >100 mmHg suprasystolic pressure. To insure arm stability and transducer placement, a customized arm rest and transducer-holder device cradled the arm and locked the transducer two to eight centimeters proximal to the antecubital fossa. B-mode images of the brachial artery were recorded from 20 seconds to three minutes after cuff release. Endothelial-independent vasodilation, the response of arterial smooth muscle cells to pharmacological doses of nitric oxide donors, was not measured because it is not associated with improvements after exercise training (22, 305) in healthy adults, administration would

interfere with the repeated measures of our main dependent variable, and previous studies using the forearm IR injury model have shown that endothelial-independent vasodilation is not affected by 20 minutes of forearm occlusion (42, 201, 241, 254, 292).

Ultrasound images were transferred to digital viewing software (Brachial Analyzer, Vascular Tools, Version 5, Medical Imaging Applications, LLC., Coralville, Iowa) where all diameters were analyzed by the same investigator (A.E.D). For each timepoint, an average of 44±3 end-diastolic diameters was analyzed before cuff occlusion (baseline diameter) and the three highest consecutive end-diastolic diameters after cuff release (maximum diameter) were used to calculate flow-mediated dilation (expressed as a percentage) using the following equation: [(maximum diameter – baseline diameter)/ baseline diameter] x 100 (75). In healthy humans, endothelium-independent vasodilation, the response of vascular smooth muscle cells to nitric oxide donors, is not associated with (22, 305) or altered by (22) exercise training. Because of this and other study design constraints, endothelium-independent vasodilation was not measured.

Blood samples were obtained from the left antecubital vein using a closed IV catheter system (Saf-T-Intima, BD Medical, Sandy, Utah) before (baseline) and three minutes after the first five minute cuff occlusion (post-FMD/pre-IR), and three, 15, 30 and 45 minutes after the 20-minute cuff was released (Figure 3.1). Serum total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides were measured at baseline using a multianalyte chemistry analyzer certified by the Centers for Disease Control's Cholesterol Reference Method Laboratory Network (Cholestech LDX System, Cholestech Corporation, Hayward, California). Baseline whole-blood viscosity was measured at 37°C at 60 rpm using a CPE-40 cone spindle and plate viscometer (DV-I+, Brookfield Engineering Laboratories, Inc., Middleboro, Massachusetts), and hematocrit

was measured using a microcapillary reader (Damon/IEC Division, Needham, Massachusetts).

STATISTICS. One-Way ANOVA was used to identify significant differences in descriptive variables and ANOVA with repeated measures (exercise group and time as "within subjects" factors) was used for determining changes in FMD. Bonferroni post-hoc tests were used to identify significant data points ($p<0.05$). In the case of missing data points, the group average (descriptive variables), group average at that timepoint (brachial FMD), or the average of timepoints before and after (brachial diameters at 15 and 30 minutes after IR injury) was substituted. Univariate correlation and multiple regression analyses were used to identify significant determinants of brachial FMD. All data are expressed as mean±SEM.

RESULTS

Endurance-trained subjects had significantly higher maximal oxygen consumption and a lower body fat percentage compared with sedentary subjects while resistance-trained subjects had higher total and lean body mass compared with sedentary subjects and lower maximal oxygen consumption than endurance-trained subjects (Table 3.1). The endurance-trained group had significantly higher brachial and ankle systolic blood pressure, ankle mean arterial blood pressure, and ankle pulse pressure than sedentary subjects though all values were within clinically normal ranges (Table 3.2). Resistance-trained subjects had lower radial and aortic augmentation indices compared with their sedentary counterparts (Table 3.2). In resistance-trained subjects, total cholesterol concentration was higher than sedentary subjects, and triglyceride concentration and blood viscosity were higher than endurance-trained subjects (Table 3.3). Resistance-trained and endurance-trained subjects consumed more total calories per day than sedentary subjects while resistance-trained subjects ingested a higher percentage of protein than endurance-trained subjects and higher amount of vitamin C per day than sedentary subjects (Table 3.4).

Ultrasound-derived measures of the brachial artery revealed that resistance-trained subjects had larger brachial artery diameters (Table 3.5) and this was reflected in most timepoints with baseline and peak diameters being higher than sedentary subjects. However, when normalized for body mass (values not reported), these differences disappeared, suggesting that a larger body size, not vascular remodeling, was responsible for the larger brachial arteries in resistance-trained subjects. Though not significantly different between sedentary, endurance-trained, and resistance-trained groups, flow-mediated dilation (FMD) measured 15 minutes after ischemia was significantly lower

than baseline when all groups were combined, suggesting that the 20-minute occlusion induced endothelial injury (Figure 3.2). The drop in brachial FMD from baseline to 15 minutes after IR injury was equally attributed to a higher baseline diameter (explaining 16% of variance), an indicator of residual dilation from the 20 minute occlusion, and a lower peak diameter (explaining 15% of variance), an indicator of a blunted dilation, making the overall change in diameter smaller (Figure 3.3). In contrast, there was no significant difference between or within groups in the amount of time to reach peak dilation after cuff release (Figure 3.4).

DISCUSSION

The primary findings of this study are as follows. First, brachial flow-mediated dilation (FMD) decreased significantly after forearm ischemia, suggesting that this surrogate "model of the heart" produces significant IR injury to the endothelium of the brachial artery. Second, these measures returned to baseline levels within 30 minutes following ischemia, illustrating the transient nature of this form of ischemia-reperfusion (IR) injury. Finally, the magnitude of injury and recovery from ischemia were not significantly different amongst young sedentary, endurance-trained, and resistance-trained subjects, suggesting that exercise training is not associated with protection from vascular IR injury.

Since MacAllister and colleagues first introduced the forearm model of ischemia in 2001 (201), over 15 articles have been published in peer-reviewed journals using this technique. By inflating a blood pressure cuff on the arm for 20 minutes, a measureable but transient injury specific to the endothelium can be produced, making this model ideal for noninvasively studying IR injury in humans. In particular, previous studies have used this model to examine the effects of and mechanisms behind ischemic (42, 201, 202) and pharmacological preconditioning (97, 132, 133, 236), remote preconditioning (200, 241, 243), and postconditioning (95, 242). Using a cross-sectional study design, we examined the association between exercise training and vascular IR injury.

Endurance exercise training has been shown to enhance endothelial function (72, 87, 135, 161), especially in models of aging and disease, through enhanced nitric oxide bioavailability, lowered oxidative stress, or other unknown mechanisms (319). In addition, endurance exercise training protects the myocardium against IR injury in animals (295, 336). In contrast, studies examining the effects of resistance exercise

training on endothelial function have been less conclusive. Isolated handgrip exercise training increased endothelial function in diseased populations (16, 168, 195) and one month of resistance training increased brachial FMD in patients who had recently experienced myocardial infarction (374). In healthy individuals, whole-body resistance exercise training was associated with (262) or decreased (8) femoral vascular resistance, but did not increase brachial FMD (54, 302). Despite the conflicting findings of resistance-training on endothelial function, it is plausible that resistance exercise training may precondition the vasculature against IR injury. When exercise intensities exceed 30% of maximal voluntary contraction (MVC), as are common during resistance exercise, blood flow through the contracting muscle is briefly disrupted, causing ischemia in downstream tissues (233, 261). As such, we hypothesized that resistance-trained and endurance-trained subjects would have attenuated vascular injury compared with sedentary age-matched controls. However, our hypothesis was not supported and this may be directly related to the age and health of the population studied.

In the present study, brachial FMD was significantly lower 15 minutes after IR injury, but returned to baseline within 30 minutes. In studies measuring beyond 15 minutes after ischemia, FMD also returned to baseline within 60 minutes (201, 202). Increases in oxidative stress and inflammation, which may lower nitric oxide bioavailability after IR injury, are likely behind the observed changes in FMD after ischemic injury. The importance of adequate nitric oxide bioavailability in preventing IR injury is supported by studies involving the infusion of activators and cofactors of eNOS, an enzyme that synthesizes nitric oxide. Endothelial dysfunction from 20 minutes of limb ischemia was prevented, and plasma nitrate concentrations (an index of nitric oxide bioavailability) were maintained with an intra-arterial infusion of an activator of eNOS, bradykinin (236). Similarly, acetylcholine-induced vasodilation was maintained even

after 20 minutes of limb ischemia when tetrahydrobiopterin (BH4), a cofactor for eNOS, was infused (254).

Possible mechanisms underlying the fall in endothelial function after injury in this model include increases in oxidative stress and inflammation, which may lower nitric oxide bioavailability by inhibiting the production and/or stimulating the degradation of nitric oxide. Indeed, twenty minutes of limb ischemia is associated with subsequent decreases in plasma total antioxidant status (254), increases in neutrophil-derived markers of oxidative stress (292) and inflammation (201), and lower arterial vasoreactivity that was attenuated with an intra-arterial infusion of vitamin C (292). Though not directly measured in the present study, adequate levels of nitric oxide bioavailability from equivalent levels of oxidative stress in both exercise and sedentary groups likely contributed to the rapid and similar recovery between exercise and sedentary groups after vascular injury. With advancing age, which is associated with higher levels of oxidative stress (38, 105, 176, 327, 350), the protective effects of exercise training may emerge. Alternatively, it is possible that exercise does not mediate protection from IR injury in healthy arteries. In support of this, eight to 12 weeks of treadmill running in healthy rats did not protect the endothelial function of coronary (348) and pulmonary (83) arteries from 45 to 90 minutes of ischemia.

To gain greater insight into the possible benefits of exercise training on IR injury and to increase the clinical significance of these findings, future studies should include older, and perhaps even diseased, populations along with measures of oxidative stress and inflammation. In addition, larger sample sizes may be needed to achieve significance, especially if endothelial function is assessed by FMD, a methodology with inherent variability. Finally, it should be noted that this forearm "model of the heart" is based upon the premise that brachial artery endothelial function reflects arterial function in the

coronary arteries (7, 364). In a landmark study by Anderson and colleagues (7), brachial artery FMD was strongly associated (95% predictive value) with gold-standard measures of coronary artery function in patients with suspected coronary artery disease. Given this, we believe that this forearm model is suitable for measuring vascular IR injury in the peripheral arteries and these results may provide insight into non-pharmacological (e.g., exercise) and pharmacological approaches for treating coronary artery disease.

In conclusion, 20 minutes of limb ischemia was associated with a transient fall in endothelial function. Sedentary, endurance-trained, and resistance-trained subjects had similar responses to IR injury, suggesting that endurance training or resistance training is not associated with protection against vascular IR injury in this young, healthy population. Despite the lack of an exercise-induced protection against acute vascular injury in young, healthy subjects, exercise training may afford protection against vascular IR injury in older or diseased adults and certainly has a well-known beneficial role in the prevention of cardiovascular disease.

Table 3.1. *Selected subject characteristics*

	Sedentary	Endurance-Trained	Resistance-Trained
Male/Female	8 / 3	9 / 2	10 / 1
Age, yr	24 ± 2	26 ± 2	24 ± 2
Height, cm	168 ± 2	172 ± 2	172 ± 2
Body mass, kg	62.0 ± 2.9	67.5 ± 2.9	76.8 ± 2.9*
Body mass index, kg/m^2	21.9 ± 0.9	22.6 ± 0.7	26.0 ± 0.7
Body fat, %	24 ± 3	13 ± 2*	17 ± 2
Lean Body Mass, kg	44.5 ± 2.4	56.8 ± 2.9*	60.9 ± 3.1*
VO_2max, $mL \cdot kg^{-1} \cdot min^{-1}$	44.7 ± 3.0	60.7 ± 2.1*†	52.1 ± 1.5

Values are mean±SEM; VO_2max, maximal oxygen consumption; *$p<0.05$ vs. sedentary; †$p<0.05$ vs. resistance-trained.

Table 3.2. *Hemodynamic and vascular measures at rest*

	Sedentary	Endurance-Trained	Resistance-Trained
Brachial SBP, mmHg	110 ± 2	119 ± 2*	115 ± 1
Brachial MAP, mmHg	79 ± 2	84 ± 1	84 ± 1
Brachial DBP, mmHg	64 ± 2	66 ± 2	62 ± 1
Brachial PP, mmHg	47 ± 1	53 ± 3	54 ± 1
Ankle SBP, mmHg	119 ± 2	134 ± 4*	127 ± 2
Ankle MAP, mmHg	83 ± 2	90 ± 2*	86 ± 2
Ankle DBP, mmHg	65 ± 1	69 ± 1	67 ± 2
Ankle PP, mmHg	55 ± 2	65 ± 3*	60 ± 2
Heart rate, bpm	59 ± 3	51 ± 2	57 ± 2
Carotid AIx, %	-8 ± 5	-9 ± 4	-16 ± 4
Aortic AIx, %	5 ± 4	-6 ± 3	-7 ± 3*
Radial AIx, %	13 ± 3	5 ± 2	2 ± 2*
cfPWV, $cm \bullet sec^{-1}$	815 ± 20	812 ± 29	850 ± 39

Values are mean±SEM; SBP, systolic blood pressure; MAP, mean arterial blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; AIx, augmentation index; cfPWV, carotid to femoral pulse wave velocity; *$p<0.05$ vs. sedentary.

Table 3.3. *Fasting blood measures*

	Sedentary	Endurance-Trained	Resistance-Trained
Total Cholesterol, mmol/L	3.6 ± 0.2	3.7 ± 0.3	4.7 ± 0.3*
LDL-Cholesterol, mmol/L	2.1 ± 0.2	2.1 ± 0.2	2.9 ± 0.3
HDL-Cholesterol, mmol/L	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1
Triglyceride, mmol/L	1.0 ± 0.2	0.8 ± 0.1	1.3 ± 0.1†
Hematocrit, %	41 ± 1	40 ± 1	42 ± 1
Blood Viscosity, cP	3.24 ± 0.16	3.15 ± 0.15	3.69 ± 0.07†

Values are mean±SEM; *$p<0.05$ vs. sedentary; †$p<0.05$ vs. endurance-trained.

Table 3.4. *Daily dietary intake*

	Sedentary	Endurance-Trained	Resistance-Trained
Calories Intake, Kcals	1590 ± 150	2341 ± 120*	2233 ± 239*
Carbohydrate, %	53 ± 4	58 ± 3	51 ± 4
Protein, %	17 ± 1	16 ± 1	23 ± 3†
Fat, %	30 ± 3	26 ± 2	26 ± 1
Vitamin A, RE	581 ± 268	863 ± 197	788 ± 112
β-Carotine, µg	585 ± 451	155 ± 72	426 ± 211
Vitamin C, mg	29 ± 11	79 ± 18	90 ± 19*
Vitamin E, mg	4.30 ± 2.2	7.14 ± 3.1	1.11 ± 0.5
α-tocopherol, mg	1.36 ± 0.5	2.32 ± 0.8	3.42 ± 0.5

Values are mean±SEM; *$p<0.05$ vs. sedentary; †$p<0.05$ vs. endurance-trained.

Table 3.5. *Ultrasound-derived vascular measures of the brachial artery*

	Sedentary				Endurance-Trained				Resistance-Trained			
	Base	15min	30min	45min	Base	15min	30min	45min	Base	15min	30min	45min
Flow-Mediated Dilation, %	5.56 ±0.62	3.59 ±0.60	4.47 ±0.83	4.94 ±0.78	6.07 ±0.71	4.04 ±0.60	4.99 ±0.96	4.86 ±0.76	5.39 ±0.76	4.45 ±0.53	4.69 ±0.57	4.67 ±0.95
Baseline Arterial Diameter, mm	3.44 ±0.17	3.54 ±0.16	3.51 ±0.15	3.50 ±0.15	3.96 ±0.22	4.00 ±0.22	4.06 ±0.22	4.09 ±0.23	4.13* ±0.13	4.14 ±0.14	4.16* ±0.13	4.20* ±0.13
Peak Arterial Diameter, mm	3.63 ±0.18	3.66 ±0.15	3.66 ±0.16	3.67 ±0.15	4.19 ±0.21	4.16 ±0.21	4.25 ±0.21	4.28 ±0.21	4.36* ±0.15	4.32* ±0.14	4.36* ±0.14	4.40* ±0.15
Δ Arterial Diameter, mm	0.19 ±0.02	0.12 ±0.02	0.16 ±0.03	0.17 ±0.03	0.23 ±0.03	0.15 ±0.02	0.20 ±0.04	0.19 ±0.03	0.23 ±0.03	0.18 ±0.02	0.20 ±0.02	0.20 ±0.04
Time to Peak Diameter, s	56.4 ±4.5	67.3 ±12.1	47.3 ±4.1	56.4 ±9.7	70.9 ±13.0	60.0 ±9.7	43.6 ±2.4	56.4 ±7.0	56.4 ±9.3	56.4 ±10.0	47.3 ±5.6	49.1 ±3.1

Values are mean±SEM; *$p<0.05$ vs. sedentary at same timepoint.

Table 3.6. Absolute difference between timepoints in flow-mediated dilation

	Sedentary	Endurance-Trained	Resistance-Trained
FMD from Base to 15min, %	-1.7±0.7	-2.0±0.7	-0.9±1.0
FMD from Base to 30min, %	-0.8±0.8	-1.1±0.9	-0.6±0.9
FMD from Base to 45min, %	-0.4±0.7	-1.2±0.5	-0.7±1.2
FMD from 15min to 30min, %	1.0±0.5	0.9±0.9	0.3±0.8
FMD from 15min to 45min, %	1.4±0.5	0.8±0.8	0.2±1.3
FMD from 30min to 45min, %	0.4±0.7	-0.1±0.7	-0.1±1.3

Values are mean±SEM; FMD, flow-mediated dilation;
No significant differences between or within groups.

Table 3.7. *Percent change between timepoints in flow-mediated dilation*

	Sedentary	Endurance-Trained	Resistance-Trained
FMD from Base to 15min, %	-30.1±12.6	-29.3±11.3	-3.6±13.2
FMD from Base to 30min, %	-14.2±15.6	-15.8±16.2	3.6±17.0
FMD from Base to 45min, %	-3.5±11.6	-25.9±14.3	6.5±27.8
FMD from 15min to 30min, %	36.3±20.5	41.2±28.8	27.9±20.7
FMD from 15min to 45min, %	58.3±20.5	29.7±34.2	41.0±35.7
FMD from 30min to 45min, %	55.8±36.6	12.2±32.4	16.5±18.9

Values are mean±SEM; FMD, flow-mediated dilation;
No significant differences between or within groups.

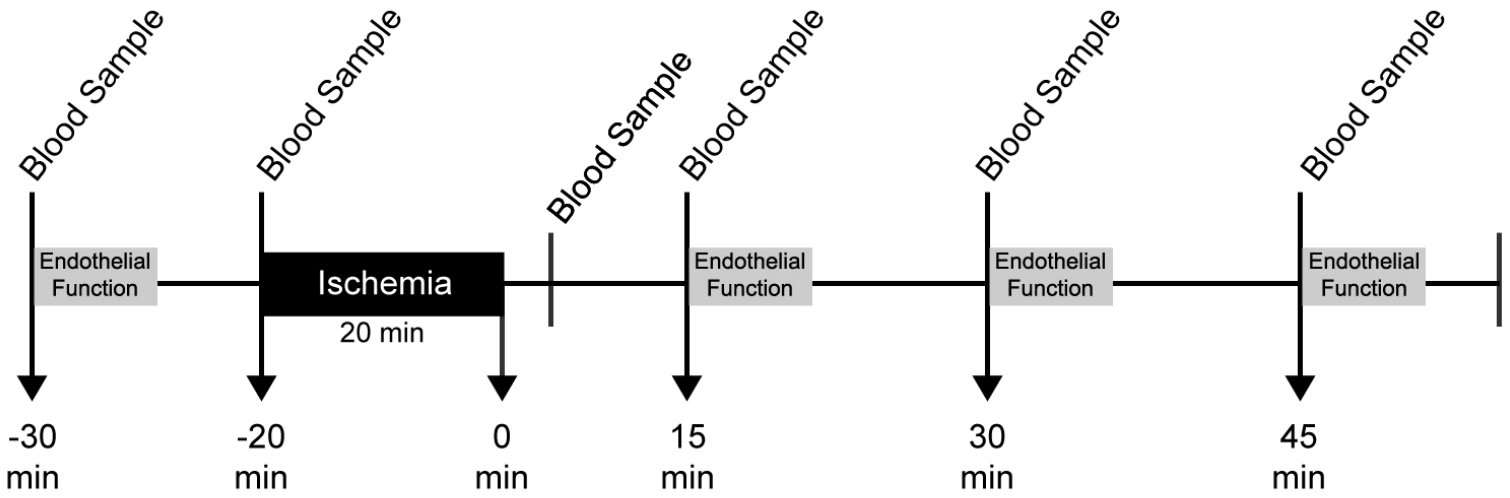

Figure 3.1. Timeline of experimental protocol.

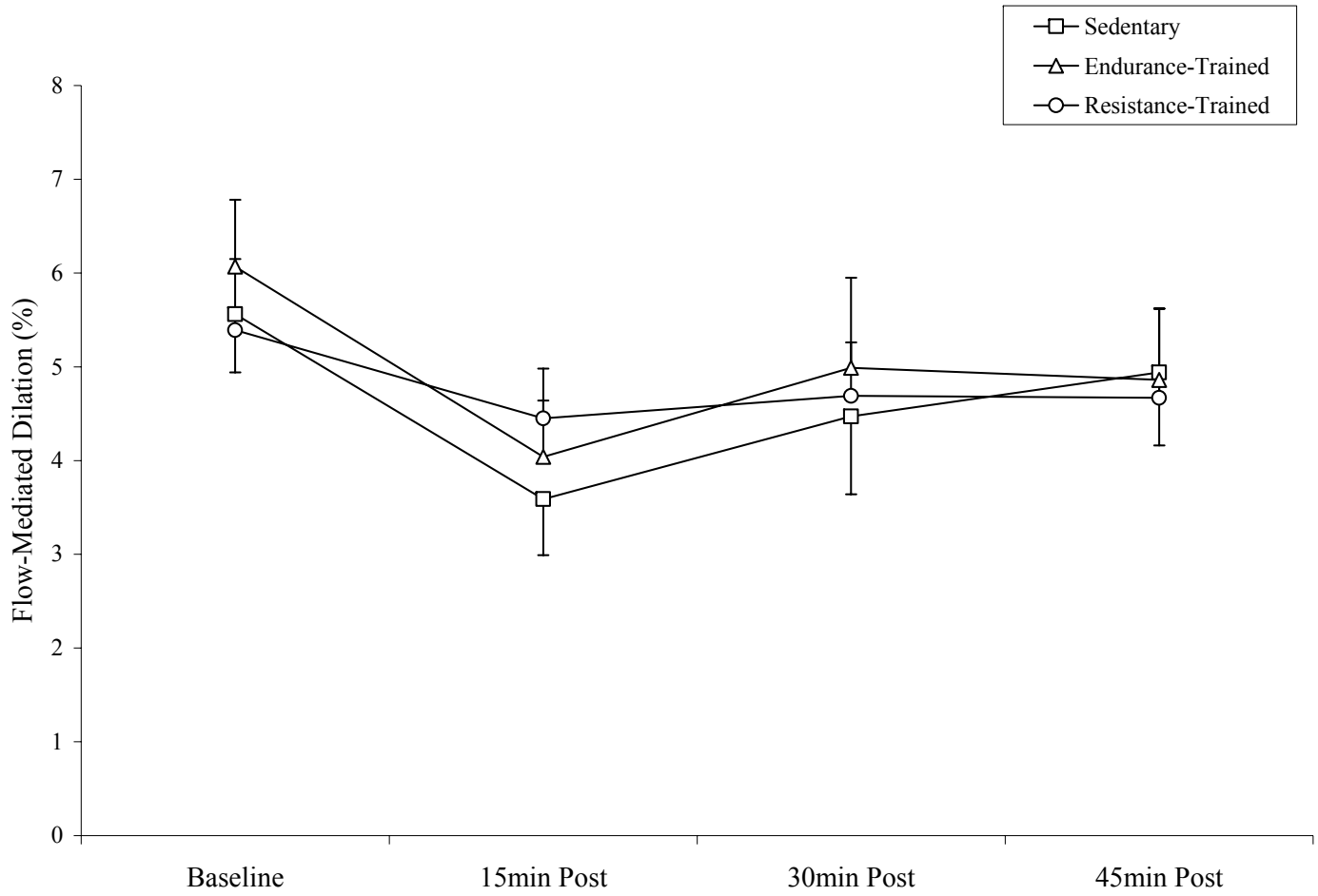

Figure 3.2. Changes in brachial flow-mediated dilation after 20 minutes of ischemia in sedentary, endurance-trained, and resistance-trained adults. There was a significant time effect at 15min Post ($p<0.05$).

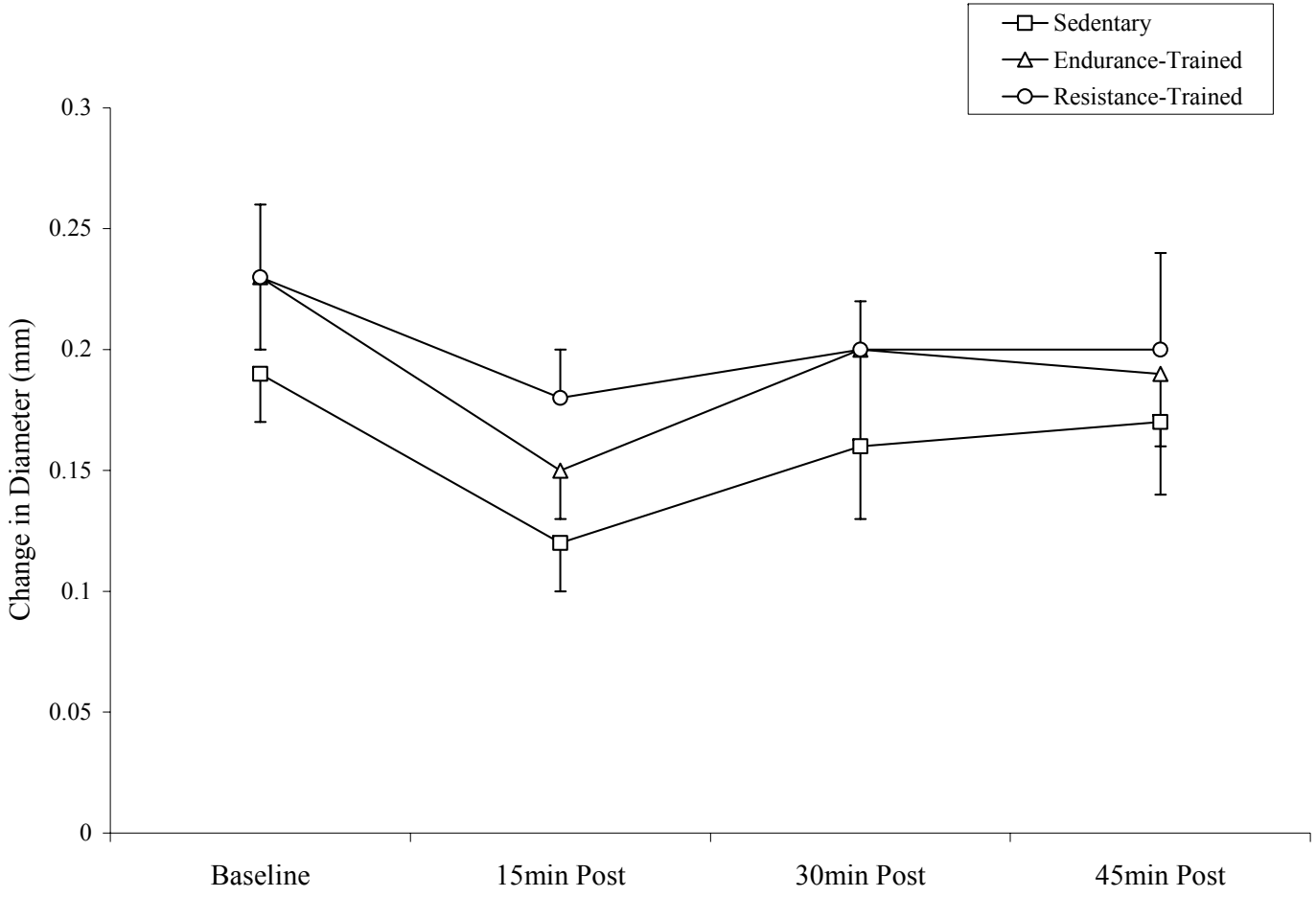

Figure 3.3. Change in brachial diameter after 20 minutes of ischemia in sedentary, endurance-trained, and resistance-trained adults. There was a significant time effect at 15 min Post ($p<0.05$).

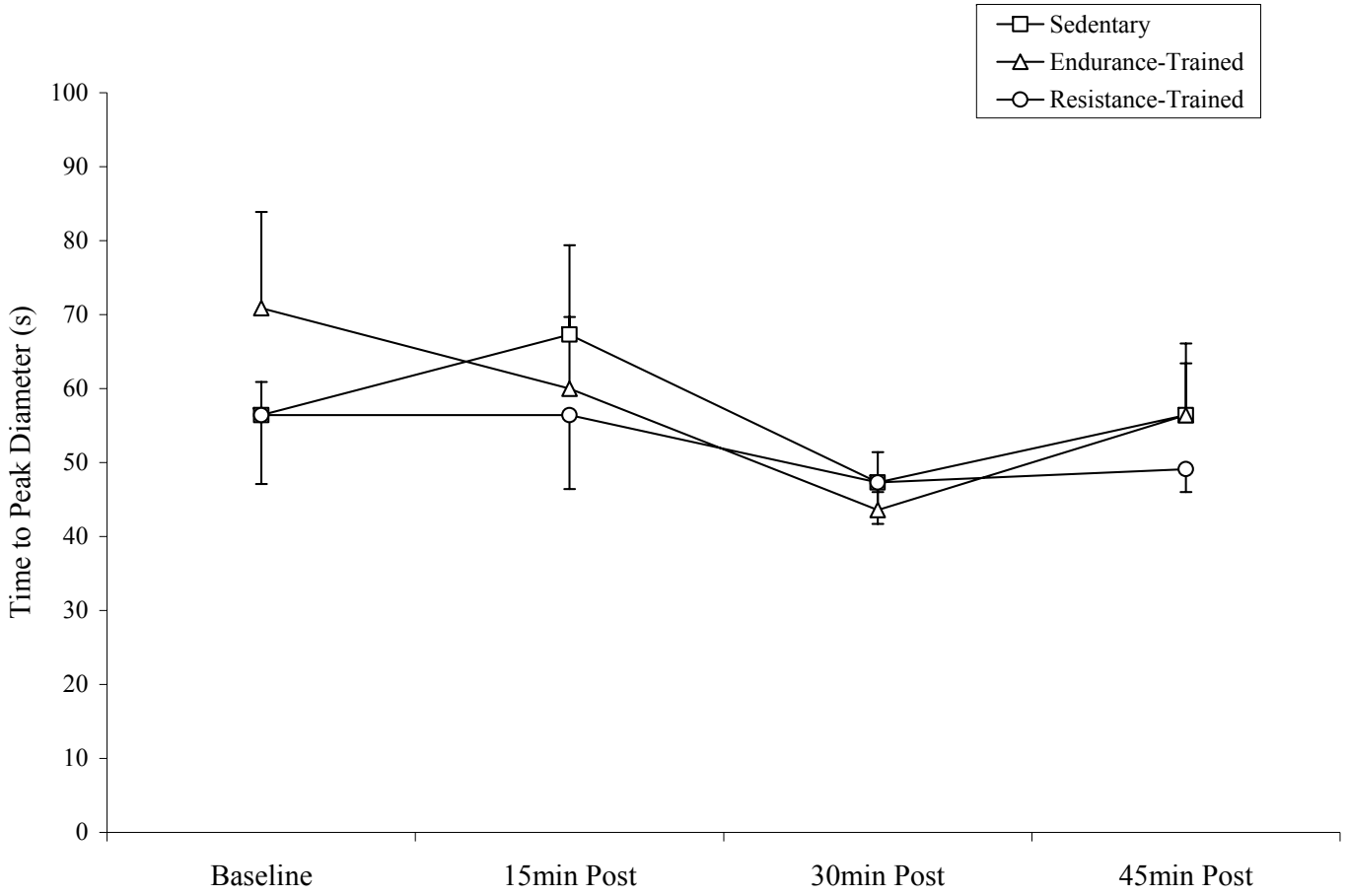

Figure 3.4. Time to peak brachial diameter after 20 minutes of ischemia in sedentary, endurance-trained, and resistance-trained adults. No significant differences between or within groups ($p<0.05$).

Chapter 4: Aging, Habitual Exercise, and Vascular Ischemia-Reperfusion Injury

INTRODUCTION

Based on data from the United States Census Bureau, it is projected that more than 71 million Americans will be >65 years of age by 2030, comprising approximately 20% of the national population (56). This, along with the rising rates of obesity, will contribute to an increase in cardiovascular-related deaths (31). Coronary heart disease, which accounts for more than half of cardiovascular-related deaths (56, 237), significantly increases the risk for myocardial infarction, which occurs when plaque build-up, blood clots, and/or vascular spasms prevent blood flow to the myocardium. Injury to the heart and vasculature from the lack of oxygen and delayed reperfusion is called ischemia-reperfusion (IR) injury. This form of injury occurs not only during a heart attack, but also during certain forms of surgical treatment for cardiovascular disease (53). Specifically, traditional coronary artery bypass graft surgery requires cardiopulmonary bypass and the introduction of arterial or venous grafts, rendering the heart and blood vessels ischemic for brief periods of time.

To prevent, treat, and understand the mechanisms behind IR injury, scientists have developed various animal models of cardiovascular disease. While these animal studies provide important mechanistic insight, they are often difficult to apply to humans because of inherent physiological differences between species. Human studies also suffer from a lack of ideal methodologies as the investigation requires ischemia of the coronary arteries. Because of this, an alternative, noninvasive, model of the heart was proposed by MacAllister and colleagues (201) that involves 20 minutes of ischemia produced by inflation of a blood pressure cuff on the human arm. Before and after cuff occlusion,

measurements of resistance and/or conduit artery endothelial function are performed. The magnitude of injury and rate of recovery from this injury are used to interpret the effects of various pharmacological and non-pharmacological treatments such as ischemic preconditioning (42, 201, 202), infusion of nitric oxide donors (97, 132, 236), and dietary interventions (96, 381).

Animal studies support the role of endurance exercise training in preventing myocardial IR injury (295, 336) and attenuating the fall in coronary blood flow after myocardial IR injury (45, 224) as well as reversing or attenuating endothelial dysfunction caused by aging and disease (178). In humans, habitual exercise has also been shown to attenuate endothelial dysfunction with age and alleviate decreases in endothelial function induced by cardiovascular disease, perhaps by increasing nitric oxide bioavailability, lowering oxidative stress, and reducing inflammation (140, 155, 218, 309). In one of the dissertation studies, we compared the brachial flow-mediated dilation (FMD), an index of endothelial function, in healthy young sedentary, endurance-trained, and resistance-trained individuals before and after 20 minutes of cuff ischemia. No significant group differences suggest that exercise training did not protect against this form of injury. However, it is possible that the low levels of oxidative stress typically found in young, healthy individuals masked the true protective effects of exercise training. Beneficial effects of exercise training may be more likely manifest in older adults with higher baseline levels. Accordingly, we studied the vascular response of both young and older endurance-trained individuals to 20 minutes of forearm occlusion. We hypothesized that after IR injury, the fall in and recovery of brachial artery flow-mediated dilation (FMD) would be similar in young sedentary and endurance-trained subjects, but that older endurance-trained subjects would recover more quickly than sedentary age-matched controls.

METHODS

SUBJECTS. A total of 45 apparently healthy adults (35 men and 10 women) aged 18 to 63 years were recruited from The University of Texas at Austin and surrounding community (Table 4.1). All subjects were normotensive (<140/90 mmHg), non-obese (body mass index <30 kg/m^2), non-smoking, free of overt cardiovascular or other chronic diseases, and were not taking any cardiovascular-acting medications as assessed by a self-reported medical history questionnaire. Exercise-training status was verified by the Godin Physical Activity Questionnaire score (129) and maximal oxygen consumption (Table 4.2). Endurance-trained subjects reported cycling and/or running >6 hours per week. The Human Research Committee at the University of Texas at Austin reviewed and approved all procedures, and written informed consents were obtained from all subjects.

PROCEDURES. Subjects reported to the laboratory twice; one session for measurements of arterial blood pressure, arterial stiffness, body composition, and maximal oxygen consumption and another session for measures of endothelial function and blood samples. For menstruating women, all vascular measures (arterial stiffness and flow-mediated dilation measures) were performed during the early follicular phase of their menstrual cycle to control for the effects of estrogen on endothelial function (57).

TESTING SESSION 1. Prior to the first testing session, all subjects were >4 hours fasted and abstained from caffeine. Body composition was measured by dual energy x-ray absorptiometry (Lunar DPX, General Electric Medical Systems, Fairfield, Connecticut), and arterial blood pressure and arterial stiffness were measured simultaneously using a validated automatic device (VP-2000, Colin Medical, San Antonio, Texas) (77). Each subject rested supine for at least 10 minutes in a quiet, dimly

lit, temperature-controlled laboratory room. Bilateral brachial and ankle arterial blood pressures were measured with the oscillometric pressure sensor method. Carotid and femoral arterial pulse waves were obtained using arterial applanation tonometry incorporating an array of 12 micropiezoresistive transducers. The femoral tonometry sensor was secured in place by a Velcro strap while the carotid sensor was held in place by a plastic collar on the common carotid artery. Aortic pulse wave velocity, a measure of arterial stiffness, and carotid augmentation index, an index of arterial stiffness and wave reflection, were measured. Pulse wave velocity was calculated from the distance (carotid to femoral artery) divided by transit time (the time delay between the carotid and femoral "foot" waveforms) (358). Carotid augmentation index was calculated as the ratio of the amplitude of the pressure wave above its systolic shoulder to the total pulse pressure as previously described (358). Radial and calculated aortic augmentation index were measured using applanation tonometry and software-derived transformation algorithms (SphygmoCor, AtCor Medical, Inc., Lisle, Illinois). Maximal oxygen uptake (VO_2max) was measured during a modified Balke incremental treadmill exercise test (1% grade increase per minute at individualized treadmill speed) as previously described (357). Oxygen consumption (indirect calorimetry via respiratory gas measurements; Physio-Dyne, Quogue, New York), heart rate, and ratings of perceived exertion (the original Borg scale) (32) were measured throughout the protocol.

TESTING SESSION 2. For the 48 to 72 hours prior to the last testing session, subjects followed and recorded a nitrate-free diet adapted from a diet created by the National Heart, Blood, and Lung Institute (273), which did not allow the ingestion of foods containing nitrates: vegetables or vegetable products, legumes, cured or processed meats, cheese, seafood or fish, alcohol, strawberries, melons, bananas, or potatoes. Adherence to the nitrate-free diet was verified by diet records, which were subsequently

analyzed by a registered dietician. Subjects did not consume vitamin, mineral, and herbal supplements >2 weeks prior to the last testing session. Testing sessions were performed in the morning to minimize possible diurnal changes in dependent variables and subjects arrived at least 10 hours fasted and >20 hours post-exercise.

Brachial blood pressure was measured two to three times using the oscillometric pressure sensor method after subjects had rested in the supine position at least 10 minutes in a quiet, dimly lit, temperature-controlled (23 to 26°C) laboratory room. Endothelial-dependent vasodilation of the brachial artery was assessed by flow-mediated dilation in the right arm before and 15, 30, and 45 minutes after 20 minutes of lower-arm cuff occlusion using an ultrasound machine (iE33, Philips Medical Systems, N.A., Bothel, Washington) equipped with a high-resolution linear array transducer as previously described (88). At each timepoint, longitudinal images of baseline brachial artery diameters were recorded proximal to the forearm cuff for an average of 90 seconds, and blood velocities were measured at 60 degrees for 30 seconds prior to forearm occlusion with a rapid cuff inflator (E20 Inflator, AG101 Air Source, and Rapid Version Cuffs, Hokanson, Inc., Bellevue, Washington) set to >100 mmHg suprasystolic pressure. To insure arm stability and transducer placement, a customized arm rest and transducer-holder device cradled the arm and locked the transducer two to eight centimeters proximal to the antecubital fossa. Ten seconds before the five-minute cuff was released, blood velocity recordings commenced and continued until 20 seconds after cuff deflation. B-mode images of the brachial artery were recorded from 20 seconds to three minutes after cuff release. Endothelial-independent vasodilation, the response of arterial smooth muscle cells to pharmacological doses of nitric oxide donors, was not measured because it is not associated with aging (55, 352) or exercise training (22, 305) in healthy adults, administration of sublingual nitroglycerin would interfere with the repeated measures of

our main dependent variable, and previous studies using the forearm IR injury model have shown that endothelial-independent vasodilation is not affected by 20 minutes of forearm occlusion (42, 201, 241, 254, 292).

Ultrasound images were transferred to digital viewing software (Brachial Analyzer, Vascular Tools, Version 5, Medical Imaging Applications, LLC., Coralville, Iowa) where all diameters and velocities were analyzed by the same investigator (A.E.D). For each timepoint, an average of 44±3 end-diastolic diameters was analyzed before cuff occlusion (baseline diameters) and the three highest consecutive end-diastolic diameters after cuff release (maximum diameters) were used to calculate flow-mediated dilation (expressed as a percentage) using the following equation: [(maximum diameter – baseline diameter)/ baseline diameter] x 100 (75). In a subgroup analysis of 17 young and 20 older subjects, the "area under the curve" (AUC) of blood velocities was analyzed during the first 15 seconds after cuff deflation (298, 299). Statistical analyses revealed no significant differences between or within groups for blood velocities ($p<0.05$). Accordingly, FMD was not normalized for shear rate or shear stress.

Blood samples were obtained from the left antecubital vein using a closed IV catheter system (Saf-T-Intima, BD Medical, Sandy, Utah) before (baseline) and three minutes after the first five-minute cuff occlusion (post-FMD/pre-IR), and three, 15, 30 and 45 minutes after the 20-minute cuff was released (Figure 1). Serum total cholesterol, LDL-cholesterol, and HDL-cholesterol were measured at baseline using a multianalyte chemistry analyzer certified by the Centers for Disease Control's Cholesterol Reference Method Laboratory Network (Cholestech LDX System, Cholestech Corporation, Hayward, California). Baseline whole-blood viscosity was measured at 37°C at 60 rpm using a CPE-40 cone spindle and plate viscometer (DV-I+, Brookfield Engineering Laboratories, Inc., Middleboro, Massachusetts), and hematocrit was measured using a

microcapillary reader (Damon/IEC Division, Needham, Massachusetts). Serum concentrations of inflammatory markers, IL-1β, IL-6, IL-8, and TNF-α, were measured before, 15 minutes, and 30 minutes after injury in young sedentary, older endurance-trained, and older sedentary subjects using a 96-well plate immunoassay (High Sensitivity Human Cytokine Kit, Millipore Corporation, St. Charles, Missouri) whereby fluorescent-coded beads bind to analytes of interest that are read by lasers (Bio-Plex Luminex 200 System, Bio-Rad Laboratories, Hercules, California).

STATISTICS. One-Way ANOVA was used to identify significant differences in descriptive variables and ANOVA with repeated measures (exercise group and time as "within subjects" factors) was used for determining changes in FMD. Bonferroni post-hoc tests were used to identify significant data points ($p<0.05$). In the case of missing data points, the group average (descriptive variables), group average at that timepoint (brachial FMD and inflammatory cytokines), or the average of timepoints before and after (brachial diameters at 15 and 30 minutes after IR injury) was substituted. Univariate correlation and multiple regression analyses were used to identify significant determinants of brachial FMD. All data are expressed as mean±SEM.

RESULTS

Young and older endurance-trained subjects had higher maximal oxygen consumption than both young and older sedentary subjects (Table 4.1). The older sedentary group had a higher body mass, body mass index than all other groups and a higher body fat percentage compared with young and older endurance-trained groups. In addition, the young endurance-trained group was leaner than their sedentary age-matched counterparts.

In general, brachial and ankle blood pressures were lower in the young sedentary group compared with the older groups (Table 4.2). For measures of arterial stiffness and wave reflection, young groups were lower than older groups, with young endurance-trained subjects having the lowest values. Serum concentrations of total cholesterol were higher in both older groups and LDL-cholesterol was higher in the older sedentary group than in young groups (Table 4.3). Both endurance-trained groups ate more calories per day than their sedentary counterparts and older endurance-trained subjects consumed greater amounts of vitamin C per day than young sedentary individuals (Table 4.4).

Brachial flow-mediated dilation (FMD) measured 15, 30, and 45 minutes after IR injury was significantly lower than baseline, suggesting that the 20-minute occlusion induced endothelial injury (Figure 4.1). For each measure of FMD after IR injury, baseline diameter (indicative of residual dilation from the 20 minute occlusion or a lack of recovery from the last FMD measurement) and peak diameter (indicative of a blunted dilation) equally explained 11 to 14% of the variability, indicating that the drop in FMD after injury was due to a combination of factors, not just a higher baseline diameter or lower peak diameter. Despite the significant fall in FMD within groups, interactions between groups were not significantly different so we conducted additional analyses

separating subjects by age (young vs. older) and exercise status (endurance-trained vs. sedentary). When subjects were compared by their exercise status, a significant time effect remained with FMD being lower than baseline values 15, 30 and 45 minutes after injury though there were no differences between groups in the magnitude or rate of recovery from IR injury (data not shown). In contrast, while both age groups demonstrated similar degrees of endothelial dysfunction after injury, the rate of recovery was slower in the older group (Figure 4.2).

Components of the FMD equation were also analyzed by ANOVA with repeated measures for significant differences within and between groups. Though there was a trend for increasing baseline diameters over time, this was not significant between groups (Table 4.5 and 4.6). The change in brachial artery diameter (peak–baseline diameter) mirrored the findings of FMD in all instances, except no significance was found within the endurance-trained group 30 minutes after injury compared with baseline (Figure 4.3). Like FMD, the change in diameter in the older group was significantly different from the younger group 30 and 45 minutes after injury. In addition, the older group took longer to reach peak diameter than the young group 30 minutes after injury (Figure 4.4). Serum cytokine concentrations revealed no significant differences between or within young sedentary, older endurance-trained, and older sedentary subjects (Table 4.7).

DISCUSSION

We tested the hypothesis that endurance exercise training in older individuals would be associated with attenuated vascular ischemia-reperfusion (IR) injury as measured by brachial flow-mediated dilation (FMD). In the present study, 20 minutes of forearm ischemia was associated with a transient fall in brachial FMD in young and older sedentary and endurance-trained subjects. Young subjects recovered more quickly from IR injury than older subjects. Specifically, 30 and 45 minutes after injury, FMD in young groups was not significantly different from baseline values whereas older groups had blunted endothelial function, suggesting that aging is associated with delayed recovery from vascular IR injury. There was no significant association between endurance exercise training and enhanced recovery from IR injury, though a trend ($p=0.179$) for a quicker recovery in older endurance-trained compared with older sedentary was observed 45 minutes after IR injury.

Animal studies demonstrate that aging is associated with increased susceptibility to myocardial IR injury resulting from alterations in cardiac gene expression, increased levels of oxidative stress, structural alterations in the mitochondria, and other unknown pathways (295). In humans, older age is the most important nonmodifiable predictor of the recovery from myocardial infarction (384). Within the vasculature, aging is also associated with detrimental structural and functional changes. Central arterial stiffness, an independent risk factor for heart disease, increases with advancing age, but is attenuated by habitual aerobic exercise, perhaps through the stretching of collagen fibers and/or alterations in arterial sympathetic-adrenergic tone (319, 359). In the present study, we confirmed these age-related findings as older subjects had higher arterial stiffness and wave reflections than young subjects. We did not, however, find lower levels of arterial

stiffness in endurance-trained subjects compared with their sedentary counterparts, which is most likely due to the small sample size and/or the use of indirect measures of arterial stiffness.

Endothelial-dependent vasodilation, a measure of endothelial function, is also reduced with age, even in healthy arteries (319). In the present study, we evaluated conduit artery endothelial function by flow-mediated dilation (FMD) in the brachial artery. Before IR injury, we did not observe a significant difference between younger and older groups in FMD, perhaps because our "older" subjects were middle-aged (49±1 yr). In contrast, age-associated differences were apparent in the rate of recovery from IR injury. While the young group's endothelial function returned to baseline within 30 minutes, the older group's values remained blunted for more than 45 minutes after injury. Thirty minutes after injury, the time to peak diameter was significantly longer in older subjects compared with young. Although we did not observe any differences in time to peak diameter before IR injury, our findings are in agreement with a previous study by Green and colleagues (23) in which the time to peak diameter was briefer in young compared with older healthy subjects. The authors speculated that the delayed time to peak diameter in older subjects may be related to increased arterial stiffness and/or increases in oxidative stress with age. Our findings of higher levels of arterial stiffness and wave reflections in older subjects support this hypothesis.

In this human model of vascular injury, the fall in endothelial function after 20 minutes cuff occlusion appears to be related to nitric oxide bioavailability, oxidative stress, and inflammation. This is supported by previous studies where the intra-arterial infusion of bradykinin (a neuropeptide that stimulates nitric oxide release) (236), vitamin C (292), or tetrahydrobiopterins (cofactors of nitric oxide and antioxidants)(254) each prevented the fall in endothelial function after 20 minutes of forearm ischemia. In

addition, plasma measures of total antioxidant status (254) and neutrophil-mediated inflammation (201) were elevated after IR injury. Because of this, we hypothesized that serum inflammatory cytokines located in the pathway upstream of neutrophils, including interleukin-1β, tumor necrosis factor-α, and interleukin-6 (169, 177), may provide insight into differential responses to the IR injury. We did not; however, find any significant differences between or within groups before or after IR injury. A lack of group differences may be related to the limited number of inflammatory markers measured and the degree of inflammation produced by 20 minutes of forearm ischemia. One previous study also found no significant differences in markers of inflammation before and after 20 minutes of ischemia (201, 202).

Despite the equivocal findings regarding the role of inflammation in this type of vascular IR injury, oxidative stress remains a primary mediator suspected in the fall in endothelial function with this model (292) and with aging in humans (105, 350). Within the vasculature, the primary sources of oxidative stress include the mitochondria, endothelial nitric oxide synthase (eNOS) uncoupling, xanthine oxidoreductase, and NADPH oxidase (266). Of these, the fall in endothelial function with advancing aging is most strongly associated with increases in the expression of NADPH oxidase and nuclear factor-κB in endothelial cells (94) and lower levels of tetrahydrobiopterin (106), a cofactor of eNOS. With this information in mind, it is possible that the delayed recovery seen in older subjects after IR injury may be due to increased basal levels of oxidative stress in the vasculature, but markers of oxidative stress were not measured in the present study. In order to determine the mechanism underlying the delayed recovery of older subjects from vascular IR injury, future studies should include measurements of oxidative stress and extend endothelial function measurements beyond 45 minutes after injury to determine the complete time course of recovery.

Another finding in this study is a lack of an association between endurance training and reduced vascular IR injury. Previous studies have shown that endurance-trained individuals have greater endothelial function than their sedentary age-matched counterparts (87, 105, 349), and an intervention study of healthy, but older subjects found increases in endothelial function after endurance exercise training (87). Therefore, we reasoned that endurance-trained subjects would recover more quickly from the 20-minute IR injury, but our hypothesis was not supported. It is possible that endurance exercise training is not associated with protection from this type of injury in humans. While exercise has been clearly shown to decrease cardiovascular morbidity and morality and improve functional capacity (124), a few studies suggest that exercise is not associated with a lower risk of recurrent myocardial infarction (282, 363), revascularization (363), or the rate of restenosis following cardiac surgery (20, 206). It is also possible that the small sample size in this study did not have sufficient power to detect significant differences between groups.

In conclusion, 20 minutes of limb ischemia was associated with a transient fall in endothelial function in young and older sedentary and endurance-trained subjects. Although there was no association between endurance exercise training and enhanced recovery from IR injury, young subjects recovered more quickly than older subjects. Within 30 minutes of injury, the endothelial function of the young group was back to baseline while blunted endothelial function persisted in older subjects for greater than 45 minutes after injury. These findings suggest that aging is associated with delayed recovery from vascular IR injury and that endurance training does not appear to modulate the vascular IR injury responses.

Table 4.1. *Selected subject characteristics*

	Young Sedentary	Young Endurance-Trained	Older Sedentary	Older Endurance-Trained
Male/Female	8 / 3	9 / 2	10 / 2	9 / 2
Age, yr	24 ± 2	26 ± 2	47 ± 1*†	51 ± 2*†
Height, cm	168 ± 2	172 ± 2	177 ± 3	176 ± 2
Body mass, kg	62.0 ± 2.9	67.5 ± 2.9	82.4 ± 3.7*†‡	70.4 ± 2.2
Body mass index, kg/m^2	21.9 ± 0.9	22.6 ± 0.7	26.1 ± 0.7*†‡	22.7 ± 0.3
Body fat, %	24 ± 3†	13 ± 2	32 ± 2†‡	17 ± 2
VO_2max, $mL \cdot kg^{-1} \cdot min^{-1}$	44.7 ± 3.0	60.7 ± 2.1*§	36.4 ± 1.6	54.2 ± 2.3*§
Godin Score, U	28.8 ± 8.9	77.0 ± 8.5*§	13.2 ± 2.3	64.1 ± 4.6*§

Values are mean±SEM; VO_2max, maximal oxygen consumption; *$p<0.05$ vs. young sedentary; †$p<0.05$ vs. young endurance-trained; ‡$p<0.05$ vs. older endurance-trained; §$p<0.05$ vs. older sedentary.

Table 4.2. *Hemodynamic and vascular measures at rest*

	Young Sedentary	Young Endurance-Trained	Older Sedentary	Older Endurance-Trained
Brachial SBP, mmHg	110 ± 2	119 ± 2	120 ± 3*	117 ± 3
Brachial MAP, mmHg	79 ± 2	84 ± 1	91 ± 2*	87 ± 2*
Brachial DBP, mmHg	64 ± 2	66 ± 2	74 ± 2*†	71 ± 1
Brachial PP, mmHg	47 ± 1	53 ± 3	46 ± 2	47 ± 2
Ankle SBP, mmHg	119 ± 2	134 ± 4	133 ± 4	143 ± 5*
Ankle MAP, mmHg	83 ± 2	90 ± 2	93 ± 2*	95 ± 3*
Ankle DBP, mmHg	65 ± 1	69 ± 1	73 ± 2*	73 ± 2*
Ankle PP, mmHg	55 ± 2	65 ± 3*	59 ± 3	70 ± 4*
Ankle-Brachial Index, U	1.1 ± 0	1.1 ± 0	1.1 ± 0	1.2 ± 0
Heart rate, bpm	59 ± 3	51 ± 2	58 ± 2	49 ± 2*§
Carotid AIx, %	-8 ± 5	-9 ± 4	14 ± 6*†	19 ± 4*†
Aortic AIx, %	5 ± 4	-6 ± 3	15 ± 3†	8 ± 3
Radial AIx, %	13 ± 3	5 ± 2	23 ± 3†	21 ± 3†
cfPWV, $cm \bullet sec^{-1}$	815 ± 20	812 ± 29	1038 ± 36*†	1038 ± 39*†

Values are mean±SEM; SBP, systolic blood pressure; MAP, mean arterial blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; AIx, augmentation index; cfPWV, carotid to femoral pulse wave velocity; *$p<0.05$ vs. young sedentary; †$p<0.05$ vs. young endurance-trained; §$p<0.05$ vs. older sedentary.

Table 4.3. *Fasting blood measures*

	Young Sedentary	Young Endurance-Trained	Older Sedentary	Older Endurance-Trained
Total Cholesterol, mmol/L	3.6 ± 0.2	3.7 ± 0.3	5.0 ± 0.2*†	4.8 ± 0.2*†
LDL-Cholesterol, mmol/L	2.1 ± 0.2	2.1 ± 0.2	3.1 ± 0.3*†	2.9 ± 0.2
HDL-Cholesterol, mmol/L	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	1.5 ± 0.1
Hematocrit, %	41 ± 1	40 ± 1	40 ± 1	40 ± 1
Blood Viscosity, cP	3.24 ± 0.16	3.15 ± 0.15	3.58 ± 0.15	3.25 ± 0.05

Values are mean±SEM; *p<0.05 vs. young sedentary; †p<0.05 vs. young endurance-trained.

Table 4.4. *Daily dietary intake*

	Young Sedentary	Young Endurance-Trained	Older Sedentary	Older Endurance-Trained
Caloric Intake, Kcals	1590 ± 150	2341 ± 120*	1888 ± 204	2434 ± 154*
Carbohydrate, %	53 ± 4	58 ± 3	50 ± 3	55 ± 1
Protein, %	17 ± 1	16 ± 1	20 ± 2	18 ± 1
Fat, %	30 ± 3	26 ± 2	30 ± 2	27 ± 1
Vitamin A, RE	581 ± 268	863 ± 197	364 ± 62	527 ± 63
β-Carotine, µg	585 ± 451	155 ± 72	177 ± 43	642 ± 112
Vitamin C, mg	29 ± 11	79 ± 18	58 ± 22	112 ± 17*
Vitamin E, mg	4.3 ± 2.2	7.1 ± 3.1	4.6 ± 1.9	3.7 ± 1.3
α-tocopherol, mg	1.4 ± 0.5	2.3 ± 0.8	11.0 ± 5.5	7.9 ± 1.9

Values are mean±SEM; *$p<0.05$ vs. young sedentary.

Table 4.5. *Ultrasound-derived vascular measures of the brachial artery in sedentary groups*

	Young Sedentary				Older Sedentary			
	Base	15min	30min	45min	Base	15min	30min	45min
Flow-Mediated Dilation, %	5.56±0.62	3.59±0.60	4.47±0.83	4.94±0.78	4.96±0.70	2.38±0.63	1.93±0.65	2.01±0.46*†
Baseline Arterial Diameter, mm	3.44 ±0.17	3.54±0.16	3.51±0.15	3.50±0.15	4.02±0.16	4.14±0.16	4.11±0.15	4.12±0.15
Peak Arterial Diameter, mm	3.63±0.18	3.66±0.15	3.66±0.16	3.67±0.15	4.21±0.15	4.23±0.15	4.19±0.15	4.20±0.15
Δ Arterial Diameter, mm	0.19 ±0.02	0.12±0.02	0.16±0.03	0.17±0.03	0.19±0.02	0.09±0.02	0.08±0.03†	0.08±0.02†
Time to Peak Diameter, s	56.4±4.5	67.3±12.1	47.3±4.1	56.4±9.7	65.0±11.8	68.3±12.7	56.7±6.9	61.7±7.2

Values are mean±SEM; *p<0.05 vs. young sedentary; †p<0.05 vs. young endurance-trained (Table 4.6).

Table 4.6. *Ultrasound-derived vascular measures of the brachial artery in endurance-trained groups*

	Young Endurance-Trained				Older Endurance-Trained			
	Base	15min	30min	45min	Base	15min	30min	45min
Flow-Mediated Dilation, %	6.07±0.71	4.04±0.60	4.99±0.96	4.86±0.76‡	5.33±0.99	3.09±0.79	2.79±0.48	3.43±0.94
Baseline Arterial Diameter, mm	3.96±0.22	4.00±0.22	4.06±0.22	4.09±0.23	4.07±0.21	4.14±0.22	4.17±0.23	4.15±0.24
Peak Arterial Diameter, mm	4.19±0.21	4.16±0.21	4.25±0.21	4.28±0.21	4.26±0.20	4.26±0.21	4.30±0.23	4.28±0.22
Δ Arterial Diameter, mm	0.23±0.03	0.15±0.02	0.20±0.04‡	0.19±0.03‡	0.20±0.03	0.12±0.02	0.13±0.03	0.13 ±0.03
Time to Peak Diameter, s	70.9±13.0	60.0±9.7	43.6±2.4	56.4±7.0	67.3±11.8	54.6±9.0	61.8±10.6	56.4±9.3

Values are mean±SEM; ‡$p<0.05$ vs. older sedentary (Table 4.5).

Table 4.7 *Absolute difference between timepoints in flow-mediated dilation*

	Young Sedentary	Young Endurance-Trained	Older Sedentary	Older Endurance-Trained
FMD from Base to 15min, %	-1.97 ± 0.53	-2.03 ± 0.70	-2.58 ± 0.59	-2.24 ± 0.79
FMD from Base to 30min, %	-1.09 ± 0.60	-1.08 ± 0.88	-3.03 ± 0.72	-2.55 ± 0.96
FMD from Base to 45min, %	-0.62 ± 0.60	-1.21 ± 0.50	-2.96 ± 0.56	-1.90 ± 0.85
FMD from 15min to 30min, %	0.88 ± 0.50	0.95 ± 0.86	-0.45 ± 0.65	-0.31 ± 0.74
FMD from 15min to 45min, %	1.37 ± 0.48	0.82 ± 0.75	-0.38 ± 0.48	0.21 ± 0.27
FMD from 30min to 45min, %	0.47 ± 0.65	-0.13 ± 0.69	0.08 ± 0.35	0.65 ± 0.82

Values are mean±SEM; FMD, flow-mediated dilation; No significant differences between or within groups.

Table 4.8. *Percent change between timepoints in flow-mediated dilation*

	Young Sedentary	Young Endurance-Trained	Older Sedentary	Older Endurance-Trained
FMD from Base to 15min, %	-30.1 ± 12.6	-29.3 ± 11.3	-43.0 ± 14.4	-33.0 ± 10.2
FMD from Base to 30min, %	-14.1 ± 15.6	-15.8 ± 16.2	-52.8 ± 20.7	-31.4 ± 11.0
FMD from Base to 45min, %	-3.5 ± 11.6	-25.9 ± 14.3	-49.8 ± 19.5	-29.5 ± 12.5
FMD from 15min to 30min, %	36.3 ± 20.5	41.2 ± 28.8	-5.5 ± 42.6	38.0 ± 35.6
FMD from 15min to 45min, %	58.3 ± 20.5	29.7 ± 34.2	0.17 ± 28.8	2.3 ± 9.3
FMD from 30min to 45min, %	55.8 ± 36.4	12.2 ± 32.4	-9.5 ± 29.2	14.3 ± 20.4

Values are mean±SEM; FMD, flow-mediated dilation; No significant differences between or within groups.

Table 4.9. *Serum inflammatory markers and adhesion molecules*

	Young Sedentary			Older Sedentary			Older Endurance-Trained		
	Base	15min	30min	Base	15min	30min	Base	15min	30min
Tumor Necrosis Factor-α, pg/mL	6.5±1.2	6.1±0.9	6.1±0.8	5.3±0.7	4.7±0.7	5.0±0.8	7.1±0.9	6.3±2.2	7.4±1.2
Interleukin-6, pg/mL	3.2±0.5	3.3±0.6	3.3±0.6	5.8±3.7	6.0±4.2	9.6±7.9	2.7±0.8	3.1±1.0	3.0±1.0
Interleukin-8, pg/mL	3.4±0.2	3.8±0.2	3.8±0.3	4.7±1.0	4.5±0.6	5.0±1.2	3.8±0.4	4.1±0.5	4.0±0.5
VCAM-1, ng/mL	1263±60	1195±66	1282±76	1369±61	1398±86	1338±63	1295±80	1341±70	1331±65
ICAM-1, ng/mL	134±12	123±11	130±13	143±13	129±11	128±10	138±12	133±11	136±10

Values are mean±SEM; VCAM-1, vascular cell adhesion molecule-1;
ICAM-1, intracellular adhesion molecule-1; No significant differences between or within groups.

Table 4.10. *Absolute difference between timepoints in inflammatory markers*

	Young Sedentary	Older Sedentary	Older Endurance-Trained
TNF-α from Base to 15min, pg/mL	-0.32±0.80	-0.49±0.50	-0.55±0.90
TNF-α from Base to 30min, pg/mL	-0.40±1.1	-0.51±0.44	0.25±1.89
TNF-α from 15min to 30min, pg/mL	-0.08±0.51	-0.01±0.37	1.72±1.56
IL-6 from Base to 15min, pg/mL	0.07±0.31	0.22±0.64	0.26±0.33
IL-6 from Base to 30min, pg/mL	0.13±0.27	3.80±4.22	0.27±0.29
IL-6 from 15min to 30min, pg/mL	0.05±0.23	3.59±3.66	0.01±0.40
IL-8 from Base to 15min, pg/mL	0.33±0.19	-0.17±0.49	0.32±0.31
IL-8 from Base to 30min, pg/mL	0.35±0.25	0.33±0.29	0.18±0.27
IL-8 from 15 to 30min, pg/mL	0.02±0.17	0.50±0.57	-0.13±0.14

Values are mean±SEM; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; IL-8, interleukin-8; No significant differences between or within groups.

Table 4.11. *Percent change between timepoints in inflammatory markers*

	Young Sedentary	Older Sedentary	Older Endurance-Trained
TNF-α from Base to 15min, %	-3.2±5.7	-10.9±4.5	-8.4±8.7
TNF-α from Base to 30min, %	-1.2±6.3	-6.3±4.9	8.9±16.2
TNF-α from 15min to 30min, %	2.7±5.0	6.1±5.7	20.5±15.6
IL-6 from Base to 15min, %	2.3±13.3	-7.1±12.0	8.2±11.9
IL-6 from Base to 30min, %	4.5±10.3	-16.9±19.6	8.1±10.2
IL-6 from 15min to 30min, %	14.0±14.8	-5.0±18.0	3.0±9.4
IL-8 from Base to 15min, %	0.3±0.2	-0.2±0.5	0.3±0.3
IL-8 from Base to 30min, %	0.4±0.2	0.3±0.3	0.2±0.3
IL-8 from 15 to 30min, %	0.0±0.2	0.5±0.6	-0.1±0.1

Values are mean±SEM; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; IL-8, interleukin-8; No significant differences between or within groups.

Table 4.12. *Absoloute change between timepoints in adhesion molecules*

	Young Sedentary	Older Sedentary	Older Endurance-Trained
VCAM-1 from Base to 15min, ng/mL	-67.8±51.1	28.8±71.5	46.0±49.9
VCAM-1 from Base to 30min, ng/mL	19.1±42.1	-31.4±49.7	36.1±62.3
VCAM-1 from 15min to 30min, ng/mL	104.4±28.6	-60.2±48.9*	-9.9±37.9
ICAM-1 from Base to 15min, ng/mL	-11.1±4.7	-14.7±7.7	-4.9±6.1
ICAM-1 from Base to 30min, ng/mL	-4.1±4.4	-14.9±7.5	-1.7±5.3
ICAM-1 from 15min to 30min, ng/mL	7.0±4.5	-0.2±5.2	3.2±3.0

Values are mean±SEM; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intracellular adhesion molecule-1; *$p<0.05$ vs. young sedentary.

Table 4.13. *Percent change between timepoints in adhesion molecules*

	Young Sedentary	Older Sedentary	Older Endurance-Trained
VCAM-1 from Base to 15min, %	-4.9±3.6	2.5±5.3	5.0±4.4
VCAM-1 from Base to 30min, %	1.3±3.3	-1.8±3.3	4.8±5.3
VCAM-1 from 15min to 30min, %	7.2±2.9	-2.8±3.6	-0.3±2.7
ICAM-1 from Base to 15min, %	-7.2±3.5	-8.8±4.5	-2.0±4.0
ICAM-1 from Base to 30min, %	-3.3±3.5	-8.9±4.0	0.9±4.3
ICAM-1 from 15min to 30min, %	4.8±3.6	1.3±4.4	3.2±2.2

Values are mean±SEM; VCAM-1, vascular cell adhesion molecule-1;
ICAM-1, intracellular adhesion molecule-1; No significant differences between or within groups.

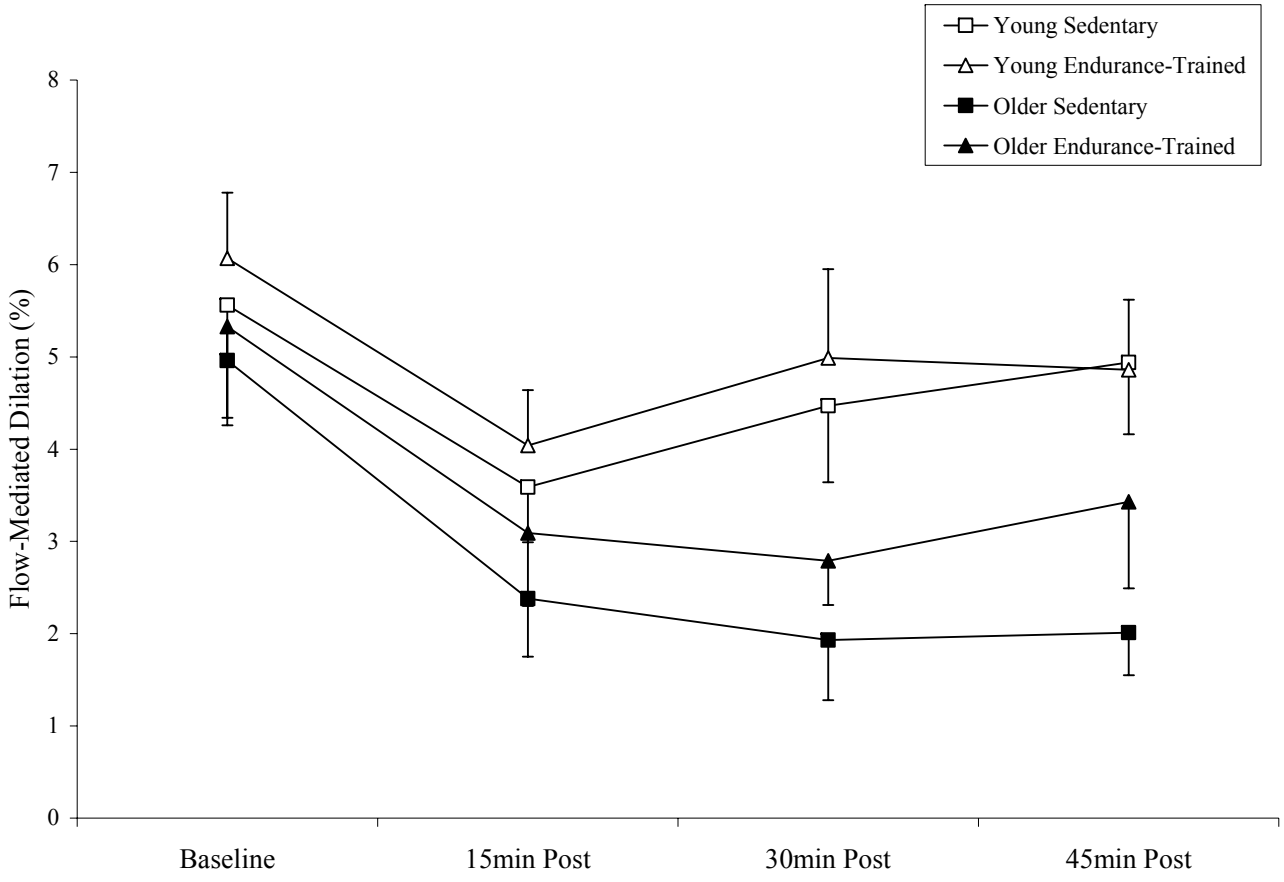

Figure 4.1. Changes in brachial flow-mediated dilation after 20 minutes of ischemia in young sedentary, young endurance-trained, older sedentary and older endurance-trained adults. There was a significant time effect at 15min, 30min, and 45min Post ($p<0.05$).

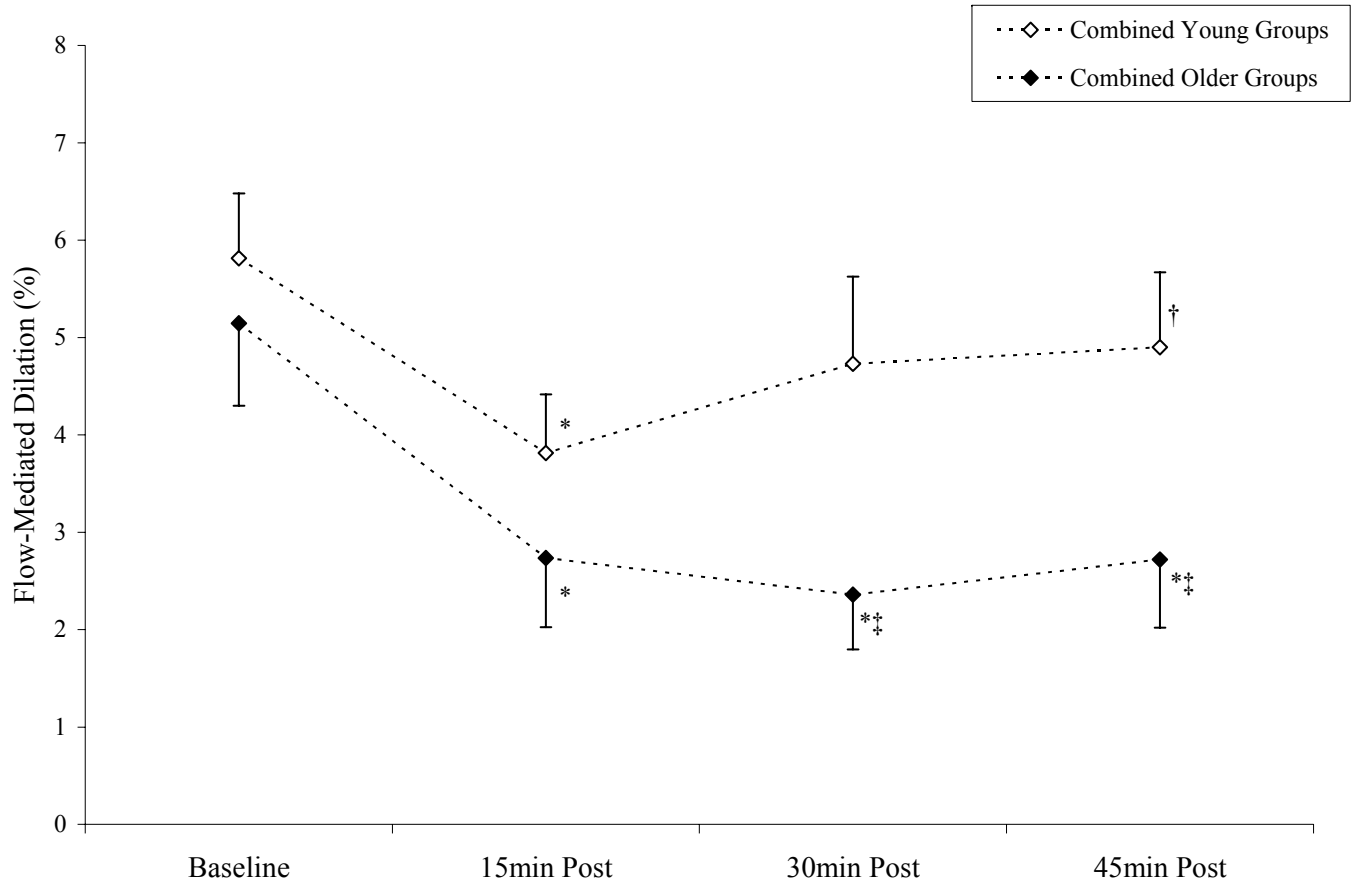

Figure 4.2. Changes in brachial flow-mediated dilation after 20 minutes of ischemia in young and older adults. *p<0.05 vs. baseline; †p<0.05 vs. 15min Post; ‡p<0.05 vs. young.

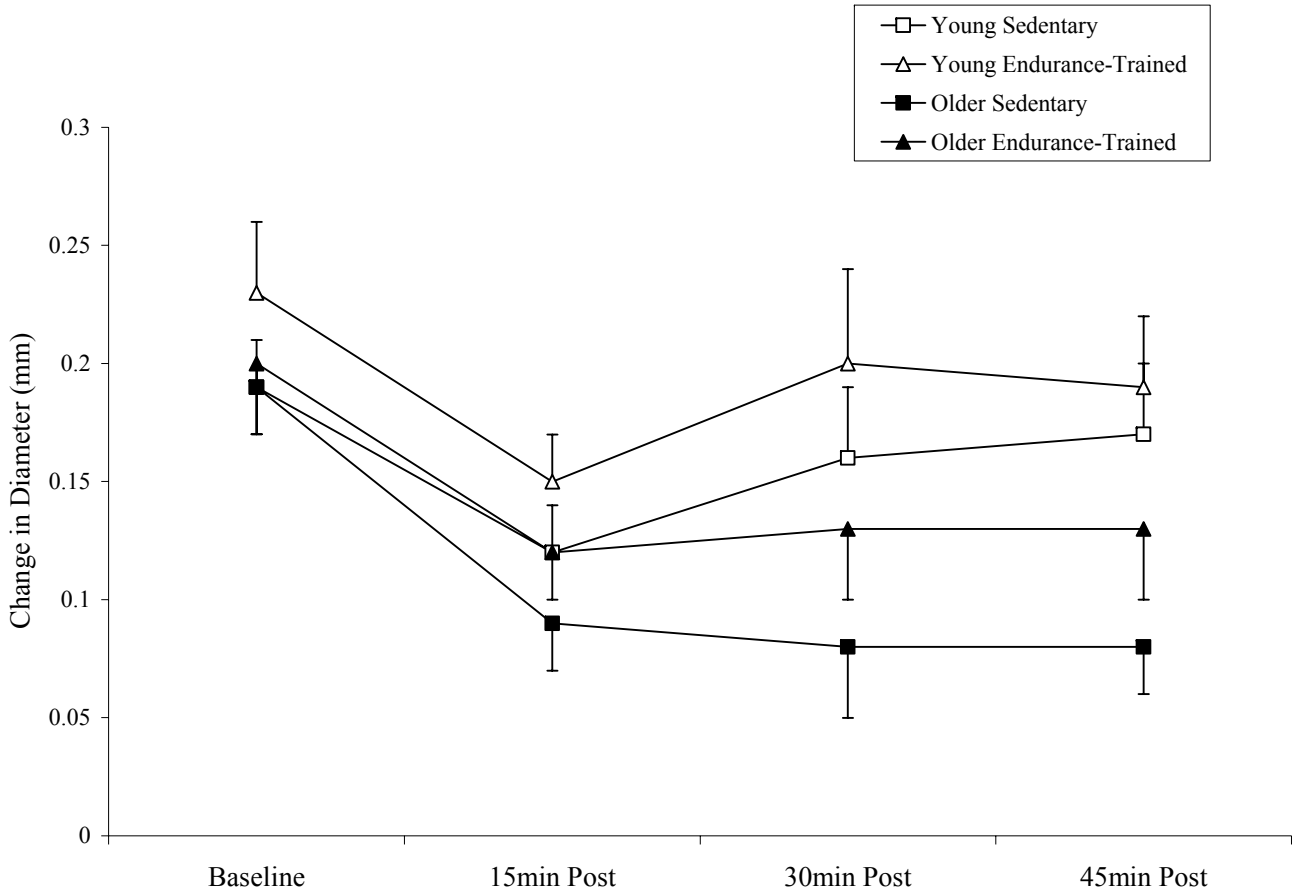

Figure 4.3. Change in brachial diameter after 20 minutes of ischemia in young sedentary, young endurance-trained, older sedentary, and older endurance-trained adults. There was a significant time effect at 15min, 30min, and 45min Post ($p<0.05$).

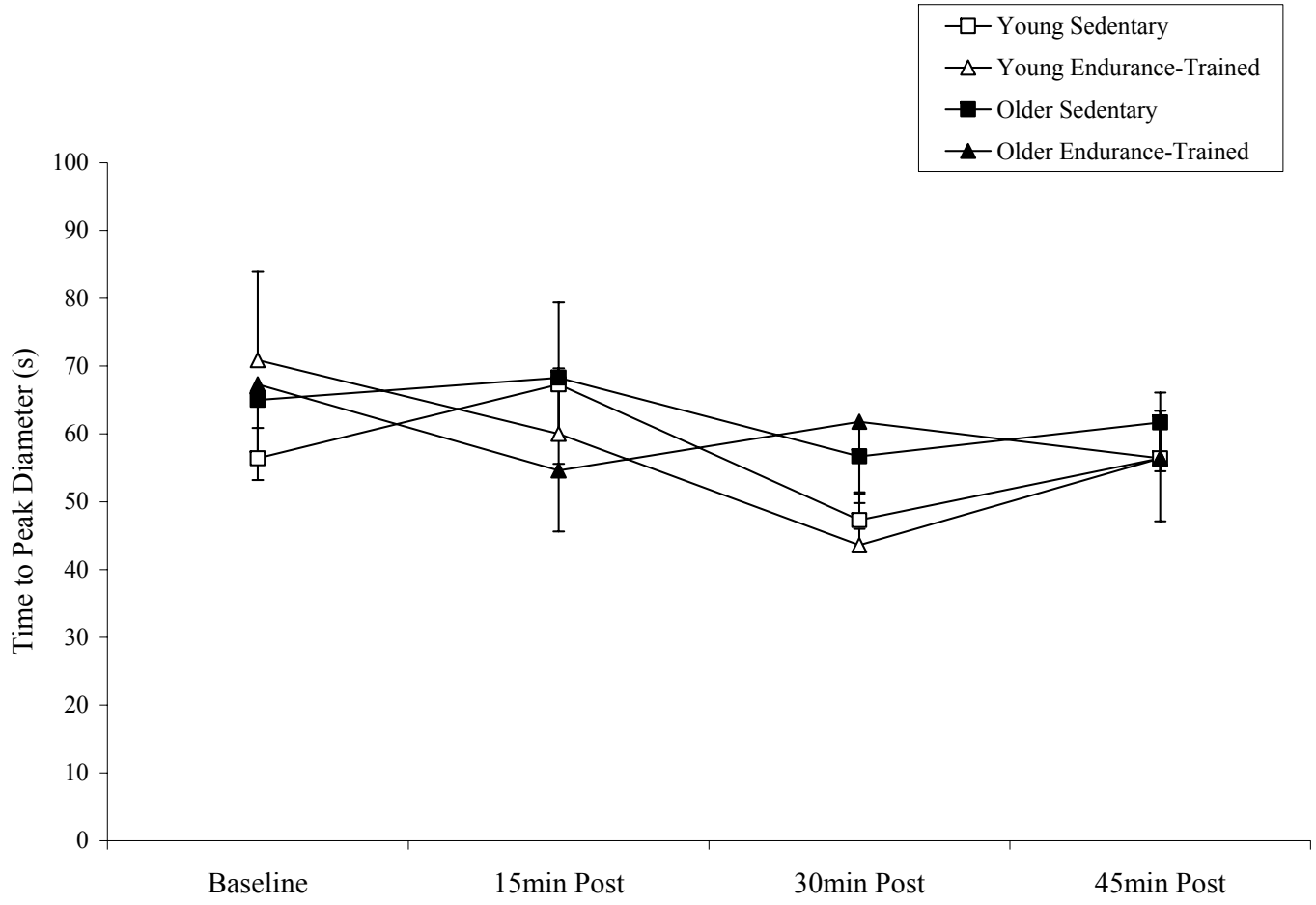

Figure 4.4. Time to peak diameter after 20 minutes of ischemia in young sedentary, young endurance-trained, older sedentary, and older endurance-trained adults.

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