ABSTRACT

Title of Document: REGULATORY EFFECTS OF ACUTE AND CHRONIC ENDURANCE EXERCISE ON NITRIC OXIDE AND REACTIVE OXYGEN SPECIES IN HUMAN CIRCULATING ANGIOGENIC CELLS

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This dissertation research comprised three studies examining the effects of acute and chronic endurance exercise on circulating angiogenic cells (CACs). Because the balance between nitric oxide (NO) and reactive oxygen species (ROS) is a critical aspect of the physiological function/dysfunction of CACs, each study determined the effects of exercise on NO-ROS balance within a variety of CAC types. Study #1 demonstrated that regular endurance exercise is associated with greater basal intracellular NO levels in cultured CACs, and that one mechanism underlying this association was increased NADPH oxidase enzyme activity in the sedentary state. Study #2 suggested an association between a sedentary lifestyle and increased nitro-oxidative stress in freshly-isolated $CD34^+$ progenitor cells. Study #3 demonstrated that prior exercise attenuates high-fat meal induced-increases in mitochondrial-derived intracellular ROS in $CD31^+$ CACs. Overall, it is concluded that acute and chronic endurance exercise enhance intracellular NO and ROS dynamics in CACs.

REGULATORY EFFECTS OF ACUTE AND CHRONIC ENDURANCE EXERCISE ON NITRIC OXIDE AND REACTIVE OXYGEN SPECIES IN HUMAN CIRCULATING ANGIOGENIC CELLS

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Chapter 1: Introduction and Specific Aims

Background

Current estimates indicate that only 50% of cardiovascular (CV) disease cases can be explained by traditional CV risk factors including hypertension, hyperglycemia, hypercholesterolemia, and others (22), and only ~60% of the CV disease risk reduction through regular exercise can be explained by its effects on traditional risk factors (151). Recent evidence indicates that the number and function of circulating angiogenic cells (CACs) may account for some of the unexplained CV risk and reduction in risk through regular exercise. The term "CACs" refers to a variety of cell types that can maintain, repair, and regenerate the vascular endothelium (250). As the development of endothelial dysfunction is thought of to be the earliest event in the development of atherosclerosis (78, 179), the maintenance of a functional endothelium by CACs is a critically important physiological process in current CV disease prevention research. Epidemiological data have established associations of CACs with CV disease risk (reviewed extensively in Chapter 2), but the biological mechanisms underlying these associations are unclear. Further, growing evidence indicates that both acute and chronic endurance exercise increase the number of CACs. This increased CAC number has been proposed to enhance the capacity for endogenous vascular repair. However, the effects of exercise or training on functional aspects of CACs have not been adequately examined.

Therefore, the overall aim of this dissertation research was to determine the effects of acute and chronic endurance exercise on molecular regulators of CAC function. The three studies contained herein examined angiogenesis- and oxidative

stress-related genes and intracellular free radicals in CACs to determine if acute and chronic exercise are associated with more favorable intracellular conditions for CAC-mediated endothelial repair. Specifically, these studies focused on the regulation of nitric oxide (NO) and reactive oxygen species (ROS), as these molecules are important mechanistic regulators of a variety of CAC maintenance and repair functions, including mobilization, homing/migratory capacity, and secretion of angiogenic growth factors (61, 88, 250). In addition, *ex vivo* experimental inhibition of NADPH oxidase was performed in each experiment to determine the mechanistic role of this pro-oxidant enzyme relative to any effects of exercise or exercise training on CACs.

Specific Aims

Specific Aim 1. To determine the effects of acute and chronic endurance exercise on intracellular nitric oxide in putative endothelial progenitor cells.

Study #1 (Jenkins et al., *Am J Physiol-Heart Circ Physiol* 2009; Chapter 3) determined the effects of acute and chronic endurance exercise on the intracellular environment of cultured endothelial progenitor cells (EPCs), which represent one type of CACs. It was hypothesized that acute and chronic endurance exercise would increase intracellular nitric oxide (NO) levels. Experiments with apocynin, an inhibitor of NADPH oxidase, were performed to examine whether alterations in NO could be explained by changes in NADPH oxidase enzyme activity.

Specific Aim 2. To determine the effects of acute and chronic endurance exercise on intracellular NO, superoxide production and angiogenic gene expression in circulating $CD34^+$ progenitor cells.

$CD34^+$ progenitor cells in the circulation provide a pool of CACs and promote vascular repair (139, 209). In Study #2 (Jenkins et al., *J Appl Physiol*, in press, 2011; Chapter 4), intracellular superoxide ($O_2^{\cdot-}$) and NO were assessed in freshly-isolated $CD34^+$ cells of trained and sedentary men before and after acute exercise. The mechanistic role of NADPH oxidase underlying any effects of acute or chronic endurance exercise on NO or $O_2^{\cdot-}$ was determined by incubating cells with or without apocynin. It was hypothesized that acute and chronic endurance exercise would favorably influence the balance between NO and $O_2^{\cdot-}$ in freshly-isolated $CD34^+$ PBMCs, and elevated NADPH oxidase activity in cells of the sedentary group would be a causal mechanism underlying these effects. It was also hypothesized that the expression of genes involved in the regulation of intracellular NO and $O_2^{\cdot-}$ levels would be consistent with greater antioxidant capacity and angiogenic function in the trained state.

Specific Aim 3. To determine the effects of endurance exercise on CACs during postprandial lipemia.

In study #3, the *in vivo* circulating environment was experimentally manipulated using a high-fat meal to induce postprandial lipemia (PPL) (Jenkins et al., *Arterioscler Thromb Vasc Biol*, submitted, 2011; Chapter 5). PPL is one of the most dramatic experimental perturbations of the CV and metabolic systems that can be performed in human subjects. PPL-induced hypertriglyceridemia has severe CV effects, including reduced NO bioavailability, increased systemic oxidative stress, and impaired endothelial function (237). However, acute exercise performed on either the preceding day (221) or during lipemia (160) can ameliorate PPL-induced endothelial

dysfunction. Importantly, PPL also causes pro-atherogenic phenotypes in circulating mononuclear cells, including upregulation of adhesion molecules and expression of proinflammatory genes (5, 153). CACs have never been examined during PPL, but exposure to an atherogenic environment promotes foam cell formation in cultured $CD34^{+}$-derived EPCs (38), suggesting that these cells may be adversely affected by elevated lipids *in vivo*. As most people in Western societies are in a postprandial state most of the time, and atherosclerosis is a disease process mediated to a large extent by postprandial events (129, 192, 264), the results of this experiment are of high clinical relevance and public health significance. It was hypothesized that (i) PPL would reduce intracellular NO levels and increase the production of ROS by NADPH oxidase and/or mitochondria; and (ii) that these effects would be attenuated by a single bout of exercise performed on the prior day.

Chapter 2: Review of Literature

Circulating Cells for Endogenous Vascular Repair

The development of endothelial dysfunction is considered to be the first step in the etiology of cardiovascular (CV) disease and is a predictor for future CV events (189). Recent research has attempted to improve the understanding of physiological, cellular, and molecular processes involved in the maintenance of a functionally-competent endothelial layer throughout the vasculature. Historically, it was thought that blood vessels lacked intrinsic mechanisms to repair cellular damage that causes endothelial dysfunction, especially damage resulting from inflammatory and oxidative injury that ultimately lead to atherosclerosis (179, 180). However, it is now known that although vascular endothelial cells have limited proliferative capacity, and are therefore unable to replace themselves to maintain a functional endothelial layer, there are several circulating cell types that perform critical functions for vascular endothelial maintenance, repair, and growth (12, 13). This dissertation research examined the effects of exercise on subpopulations of peripheral blood mononuclear cells (PBMCs), termed circulating angiogenic cells (CACs), with known roles in vasculogenesis and established relationships with endothelial function and CV disease risk. This chapter will review the current understanding of (i) the definition and functions of CACs, (ii) the relationship between CAC number and function and CV disease risk, (iii) cellular and molecular mechanisms linking CACs and CV disease, and (iv) the effects of lifestyle factors (i.e, physical activity an dietary habits) on CAC number and function.

Definitions and Functions of CAC Types

Overview. Circulating angiogenic cells (CACs) are PBMCs that perform a variety of functions in maintaining the endothelial lining of blood vessels. CACs are thought to support blood vessel maintenance in at least one of the following ways: by migration to sites of endothelial damage and incorporate into the endothelial monolayer, thus providing a 'fresh' endothelial cell to replace a damaged one; contributing to angiogenesis by incorporating into new vessels as they sprout/expand into tissues requiring increased perfusion; and/or via the release angiogenic growth factors/cytokines, such as VEGF, in a paracrine manner at sites of new vessel growth or endothelial damage, aiding in recruitment and proliferation of endothelial cells or other CACs. For this literature review, the term "CAC" is used in a broad sense to refer to any cell type previously shown to perform one or more of these functions. Current data indicate that these cells comprise at least four CAC subpopulations: (i) endothelial progenitor cells (EPCs); (ii) circulating bone marrow-derived $CD34^+$ progenitor cells (regardless of coexpression of an endothelial antigen; (iii) pro-angiogenic hematopoietic cells; and (iv) circulating endothelial cells that originate from the vessel wall.

Endothelial Progenitor Cells. Endothelial progenitor cells (EPCs) originate early in embryonic development where they are derived from a common mesenchymal stem cell that gives rise to the hematopoietic and endothelial lineages (termed the "hemangioblast") (95, 96, 175). Until recently, it was thought that EPCs existed only in the prenatal environment in mammals, and angiogenesis during adulthood was believed to result from the sprouting of existing vascular endothelial cells to form

new blood vessels (175, 176). However, in 1997 Asahara et al. (13) discovered EPCs also exist as a rare subpopulation of PBMCs in humans that co-express a hematopoietic stem cell marker (CD34) and an endothelial marker. These cells had the capacity to re-endothelialize denuded vessels and contributed to *de novo* vasculogenesis in vivo (13). Thus, the authors identified an entirely new mechanism for the maintenance of vascular integrity in adults, and the clinical implications of this discovery of circulating cells with vasculogenic capacity have become increasingly recognized in recent years.

EPCs contribute to repair of the endothelium by direct (i.e., engraftment) and indirect (i.e., paracrine secretion of angiogenic factors) mechanisms (26, 167). The exact degree to which EPCs perform endothelial repair by engraftment in damaged vessels is a controversial issue and has been found to be variable across several studies from animal models. One study found that 30% of endothelial cells were derived from progenitor cells in an allogeneic transplant model (97). Effect sizes in other studies have been smaller, ranging from ~10% (34, 210) to less than 5% (63); furthermore, several studies have found no evidence for EPC engraftment into the endothelium under physiological conditions (75, 76, 168, 238). This variability in results among published studies is probably related to the different experimental approaches among laboratories. The studies using severe ischemia models have most consistently supported a role for EPCs in formation of new blood vessels. In this experimental approach, a major vessel (usually the femoral artery) is ligated and the downstream tissues are subjected to a period of ischemia. The ischemic tissue becomes necrotic and the animal may lose the affected hindlimb. However, studies in

which ischemic tissues have been treated with EPCs have prevented the ischemia-induced necrosis by promoting vascularization and restoring tissue perfusion (4, 11, 186, 253). Data from these studies have been largely interpreted by the field as evidence for a role of EPCs in growth of new vessels (225). However, the hindlimb ischemia method is a severe experiment and its relevance to the physiological/clinical situation is unclear; i.e., there are no diseases (even peripheral arterial disease with severe ischemia) in which human patients experience an ischemic stimulus as dramatic and sudden as is seen in the mouse hindlimb ischemia model. A proposed alternative mechanism for EPC-mediated vascular maintenance is secretion of growth factors in a paracrine manner, and there is growing experimental evidence supporting this notion (85, 116, 263). Thus, the current thinking is that the major contribution of EPCs to the maintenance of vascular integrity is through pro-angiogenic functions, and that engraftment probably plays a minor role (85, 217) except in cases of severe tissue ischemia.

Issues with EPC terminology based on cell origin and phenotype. It has recently become clear that a number of cell types are involved in the maintenance of endothelial integrity, and the term "EPC" is now considered a broad term and may apply to a number of specific cell subpopulations. Broadly speaking, two functionally distinct subpopulations of EPCs have been identified. Early EPCs organize into colonies in culture after ~1 wk, promote vascular repair by secretion of pro-angiogenic growth factors at sites of endothelial injury, but have a low capacity to self-replicate and do not contribute to the formation of new vessels in vitro or in vivo (27, 92, 105, 260, 261). Conversely, late EPCs form colonies after an extended time

in culture (~2-4 wk), have robust proliferative capacity and contribute directly to neovascularization (72, 105, 144, 217, 261). It is extremely controversial whether EPCs of any particular subset or origin constitute “true” EPCs. Briefly, the confusion arises partly from the fact that there are additional circulating mononuclear subpopulations with angiogenic potential displaying substantial overlap with EPCs in their origin and phenotype. For example, some subpopulations of T-cells and monocytic macrophages co-express endothelial antigens and perform angiogenic functions (discussed below – see *Angiogenic Monocytes and T-Cells).* For these reasons, it has been suggested that the term “EPC” should be avoided unless the terms “endothelial” and “progenitor” can be defined using sophisticated methodologies to determine expression of specific cell surface markers and *in vivo* vasculogenic potential (92).

Circulating CD34+ progenitor cells as CACs. Despite the widespread disagreement and controversies over cell definitions in the CAC field, cells expressing the hematopoietic stem cell antigen CD34 have been shown to contain progenitor cell subsets with angiogenic function. The presence of one or more endothelial markers, e.g. the vascular endothelial growth factor (VEGF) receptor-2 (VEGFR2) [as originally reported by Asahara (13)], CD144, CD146, CD31, von Willebrand factor, and others have been used to identify EPCs from within the $CD34^+$ cell pool (92, 105, 217). However, $CD34^+$ cells are scarce in the adult human population [< 1% of total PBMCs, (184, 185)]. Thus, for practical purposes, the $CD34^+$ population, without further subdivision into subsets expressing endothelial antigens, is often used for CAC studies (15, 42, 184, 254). Human and animal cell

therapy studies using autologous transplantation of $CD34^+$ cells have found beneficial CV-related outcomes (89, 90, 98, 262), providing proof of concept for the angiogenic potential of circulating $CD34^+$ cells. Recent reports indicate that $CD34^+$ cell numbers are lower in patients with CV and metabolic diseases compared to healthy populations (55, 56), further supporting a link between $CD34^+$ cells and systemic CV pathophysiology. However, it is unlikely that inclusion of only the CD34 antigen is sufficient for enumeration or functional analysis of CACs. This is because of the heterogeneity of $CD34^+$ subpopulations as discussed above, and also because of the existence of a number of non-progenitor hematopoietic cells that can function as CACs, which will be described in the next section.

Angiogenic monocytes and T-cells. Although most of the research on CACs has focused on the angiogenic actions of progenitors, there are also developmentally mature (i.e., $CD34^-$) hematopoietic cells that co-express endothelial antigens and perform angiogenic functions. There is a large body of work on monocytes involved in angiogenesis, as monocytes have long been known to adhere to the endothelial layer of vessels undergoing collateral growth (182). Pharmacologic inhibition of monocyte recruitment from the bone marrow substantially reduced angiogenesis, suggesting that monocyte recruitment is indeed required for growth of new vessels (86). Although the mechanism by which monocytes support angiogenesis is not completely clear, there is evidence that monocytes become activated by signals, e.g. monocyte chemoattractant protein-1, secreted from the cells at the edge of vessels undergoing angiogenesis (106). These activated monocytes in turn secrete angiogenic growth factors such as VEGF to aid in further angiogenesis (9). This process requires

leukocyte-endothelial cell interactions that are mediated by the glycoprotein CD44 (234) and monocyte invasion into the endothelium, which is coordinated by expression of adhesion molecules known as integrins (84). In addition, recent evidence indicates that monocytes are an exogenous source of nitric oxide (NO) for the vascular endothelium during angiogenesis (219). Particularly interesting was the finding that the NO synthase (NOS) isoform required for angiogenesis was the inducible NOS (iNOS) isoform, not the endothelial (eNOS) isoform (219), which is the major source of NO involved in endothelial cell-dependent vasodilation (102). These data came from experiments with iNOS and eNOS knockout mice, and only iNOS knockout mice had impaired angiogenic activities. However, the relative contributions of iNOS and eNOS to human CAC function in the context of vascular maintenance or disease-related impairments have not been investigated.

The term “monocyte” is a general term referring to a number of different PBMC subpopulations involved in immune function, tissue inflammation, and host defense. The question of which monocyte cell fractions are responsible for angiogenic functions has received remarkably little attention in recent years, as the field has mostly focused on CACs that can be defined specifically as “EPCs” and not on other cells previously known to aid in angiogenesis. The monocytic $CD14^{+}/CD34^{-}$ cell fraction has been reported to take on an endothelial cell phenotype in angiogenic culture conditions (79, 188). Indeed two separate publications have concluded that $CD14^{+}/CD34^{-}$ (79) or $CD14^{+}/CD34^{low}$ (178) cells are the primary source of human CACs. These cells have been shown to cooperate with $CD34^{+}$ cells in angiogenesis (8, 79), suggesting that interactions among CAC types are important to consider.

Furthermore, some EPCs (i.e. early EPCs) display phenotypic overlap with monocytes/macrophages under certain conditions (e.g., phagocytosis of invading bacteria) (173, 261). Monocytic progenitors from bone marrow have been shown to adhere to injured endothelium and accelerate re-endothelialization by adopting an EPC phenotype (64). A population of monocytes has been reported to patrol healthy endothelium, recognize inflammation, and remove inflamed tissue by initiation of the early immune response (14). Together, these data suggest the existence of one or more populations of circulating angiogenic monocytes that display a high degree of functional plasticity and are probably an under-appreciated source of endothelial vascular repair.

Similar to the case for monocytes, evidence has existed for many years that T-cells are involved in angiogenesis. Over 20 years ago, T-cells were reported to produce a "lymphocyte angiogenesis factor", and the degree of this angiogenesis factor secretion was found to decline with age (73). Recent evidence also indicates that angiogenic T-cells can home to sites of endothelial injury and secrete angiogenic growth factors in a manner similar to other CACs (43, 100, 120). Kushner et al. found that $CD31^+$ T-cells secrete angiogenic growth factors in large quantities (120) and that their capacity to do so declines with age (122). In addition, data from studies on EPCs, specifically early EPCs, have implicated a role for T-cell subpopulations as CACs. Microarray gene expression analysis indicated that the commonly-used early EPC colony-formation assay (91) consists of hematopoietic cells with a gene expression signature similar to that of T-cells, but having little genomic resemblance to endothelial cells (41). Furthermore, depletion of T-cells before plating prevented

formation of CAC colonies from monocytic cells (227). Therefore, the role of angiogenic T-cells as CACs for endothelial repair has also been inadequately investigated.

Circulating endothelial-like cells. The vessel wall itself has been implicated as a source of CACs (104, 259). Endothelial cells lining the vessel wall are generally considered to be fully differentiated cells with limited proliferative potential (20). However, using single cell assays for proliferative and clonogenic capacity, Ingram et al. showed that human aortic endothelial cells contain a complete hierarchy of highly proliferative progenitor cells (104). Thus, since these cells are sloughed from the endothelial lining (225), the endothelium can act as its own source of CACs for tissue renewal. Furthermore bone marrow (114) and circulating (115) cells expressing the endothelial antigen CD31 but lacking the progenitor antigens (e.g. CD34 or cKit) have been described as having profound angiogenic potential. Several authors (34, 114, 115) emphasized a number of important implications for the existence of proangioegenic $CD31^+$ endothelial-like cells for cell-mediated angiogenesis: (i) $CD31^+$ cells are far more abundant in peripheral blood of adults than progenitor cells, indicating CACs may be more numerous than commonly thought (79); (ii) because of this high cell frequency, $CD31^+$ cells have the advantage of not requiring ex vivo expansion to obtain significant numbers, thus making them a potentially attractive therapeutic source of cell therapy for CV disease; (iii) one of these studies (114) showed that in bone marrow, CD31 may be a specific single marker identifying CACs for angiogenic potential, which, if confirmed, would greatly simplify the controversial issues plaguing the CAC field surrounding cell identity.

Associations between CACs and CV Disease Risk

CV disease and CAC number. Some estimates indicate that conventional CV risk factors such as hypertension and dyslipidemia explain only ~50% of CV disease cases (22), and there has been intense investigation to determine whether novel risk factors can be identified as targets for CV disease prevention and treatment. CACs are increasingly thought of as one such novel risk factor, and recent research has established solid evidence for a relationship between CAC numbers and CV disease incidence and risk. For example, the concentration of CACs are independently inversely related to endothelial dysfunction in coronary artery disease patients (249). CAC number is also related to a number of conventional CV risk factors and CV complications, including age (93), composite Framingham risk score (91), obesity (137), hypertension/pre-hypertension (136, 255), and glucose intolerance and diabetes (52), inflammation (243, 244) and the prothrombotic state (244). CACs are also reduced in overt diseases, including coronary artery disease (195), peripheral arterial disease (39), arterial calcification (31); abdominal aortic aneurysm (25), heart failure (62), and diabetes (56, 57), and atherosclerosis (54).

One possible mechanistic explanation for reduced cell numbers in these disease states is that reserves of CACs within bone marrow and the vessel wall become depleted over the normal course of aging (10, 185, 236, 241, 248). It is thought that CV and metabolic disease risk factors (e.g. hyperglycemia or hyperlipidemia, which will be discussed in detail below) continually damage the vascular endothelium and thus create a constant need for CACs, eventually resulting in the depletion of the pool of cells available for endothelial regeneration (63, 83, 99, 205, 236).

CV disease and CAC function. CV diseases and risk factors are also associated with reduced CAC function. Bone marrow transplantation experiments in young and old mice indicated an age-related decline in progenitor cell-mediated neovascularization (205). Cells from young donors injected into ischemic hindlimbs of older mice completely prevented ischemic injury, however cells from older donors had no protective effect when injected into the ischemic limbs of young mice. There is evidence that CAC number is reduced with age and in the presence of disease due to reduced capacity to mobilize cells from their niche in bone marrow and vasculature, and also due to depleted reserves (205, 241). Studies have documented an association between age and impaired functional aspects of CACs, including reduced migratory capacity (93), reduced secretion of cytokines (122), and increased apoptotic susceptibility (122). CACs from patients with metabolic and CV diseases and/or CV risk factors have exhibited a variety of impaired functional capacities (e.g., impaired migratory capacity, neovascularization potential, *in vitro* angiogenesis, etc.), (83, 87, 88, 99, 112, 113, 228).

Therefore, there is ample correlational evidence supporting a link between CV risk factors or overt disease and reduced number and function of CACs. However, investigations into the mechanisms underlying these correlations have only recently begun to appear in the literature. A summary of the current information relative to the known mechanisms of CACs related to CV disease development and pathogenesis follows in the next section.

Mechanistic Studies Linking CACs and CV Disease

In the last few years it has become increasingly clear that NO levels in CACs are critical for a number of cellular functions (61), and the molecular regulation of NO in CACs is somewhat similar to the mechanisms regulating vessel wall endothelial cell NO levels. This section will deal with mechanistic aspects of the relationship between CACs and CV disease, with particular focus on NO and oxidative stress.

Nitric oxide. NO is mechanistically involved in a number of CV functions, including smooth muscle cell relaxation for vasodilation, inhibition of leukocyte and platelet adhesion, and a number of anti-atherogenic biochemical actions (159). Recent evidence supports a critical role for NO in CAC-mediated effects on the vascular endothelium (61). The first study highlighting the importance of eNOS-derived NO in CAC biology showed that eNOS knockout mice had impaired CAC mobilization from the bone marrow and reduced CAC-mediated endothelial repair compared to wild type mice (4). Heiss et al. recently demonstrated the importance of NO as a regulator of human CAC functional capacity (88). These authors first observed that CACs from coronary artery disease patients had no detectable levels of eNOS protein whereas CACs from controls had substantial eNOS expression. The disease-associated eNOS deficiency was correlated with substantially impaired VEGF-stimulated migratory capacity compared to disease-free control subjects. However, treatment of CACs from the patient group with a NO donor completely eliminated the difference in migratory capacity between the groups, indicating that the disease-related impairment in CAC function is entirely NO-dependent.

What causes the reduced NO production in disease and in the presence of risk factors for CV disease? Accumulated oxidative stress insults are generally accepted as a principle cause of cellular aging (33, 77). Evidence from vascular endothelial cell biology suggests that oxidative stress, i.e., an imbalance between the production of reactive oxygen species (ROS) and the capacity for the cellular antioxidant defense systems to protect against excessive ROS, could be involved in CAC functional decline with aging and in disease states. However, it has only been in the last few years that careful examinations of NO- and oxidative stress-related pathways in CACs have begun to appear in the published literature. CAC oxidative stress occurs as a result of overproduction of ROS by NADPH oxidase, xanthine oxidase, and/or mitochondria (61). The principle components of the antioxidant defense system are the superoxide dismutase [cytosolic superoxide dismutase 1 (SOD1) and mitochondrial superoxide dismutase 2 (SOD2)], which convert superoxide ($O_2^{\cdot-}$) to hydrogen peroxide; catalase, which converts hydrogen peroxide to oxygen and water; and glutathione peroxidases, which prevent the oxidation of cellular macromolecules (e.g. lipids and proteins).

NADPH oxidase. The pro-oxidant family of enzymes called NADPH oxidases has been most widely studied as a source of oxidant stress in cardiovascular tissues. The NADPH oxidase (Nox) family consists of 7 homologues, 4 of which are expressed in the cardiovascular system (Nox1, Nox2, Nox4, and Nox 5) (123). There is a well-established role for Nox2 (a.k.a., $gp91^{phox}$) as the major Nox homologue in vascular endothelial cell physiology and pathophysiology (28, 47, 111, 172). In addition, $gp91^{phox}$ is expressed and plays an important role in cells with tissue repair functions

including fibroblasts, hematopoietic stem cells, and a number of CAC types (17). Therefore, this literature review and the work presented in this dissertation focus specifically on this Nox isoform and the supporting subunits with which it associates. Structurally, the catalytic subunit $gp91^{phox}$ forms a membrane-bound complex with the $p22^{phox}$ subunit. Upon activation of intracellular signaling pathways by factors such as angiotensin II (and others, discussed below) the $p47^{phox}$ subunit is phosphorylated by proline-directed kinases or protein kinase-C (49), resulting in a conformational change that facilitates translocation to the membrane from the cytosol. Through additional phosphorylation events, the $p67^{phox}$ subunit binds to the translocated $p47^{phox}$, which provides a binding site for the activated small GTPase Rac. Complete assembly of this entire subunit complex is required for activation of the enzyme. Once assembled, $O_2^{\cdot -}$ is generated via the transfer of electrons from NADPH to molecular oxygen. Low levels of NADPH oxidase activity produce $O_2^{\cdot -}$ in controlled amounts, and under normal physiological conditions $gp91^{phox}$-derived $O_2^{\cdot -}$ can act as signaling molecules for a number of biological processes, including angiogenesis (218), endothelium-dependent vasodilation (18), mobilization of CACs (223), and the functional responsiveness of CACs to growth factors (226). However, when produced in excess, NADPH oxidase-derived $O_2^{\cdot -}$ damages cellular components, and can interact with NO to produce peroxynitrite (an extremely reactive and potent source of nitro-oxidative damage) (159). NADPH oxidase overproduction of $O_2^{\cdot -}$ can also cause eNOS enzyme uncoupling, resulting in the production of $O_2^{\cdot -}$ by eNOS itself (159). Several factors associated with the development of CV disease have been shown to increase NADPH oxidase activity in endothelial cells and CACs,

including oxidized LDL-cholesterol (oxLDL) (74, 135); oxidized fatty acids (29); angiotensin II (103); hyperglycemia (50, 242), advanced glycation end products (AGEs) (245); and a number of inflammatory cytokines such as C-reactive protein (170), TNFα (148), and proinflammatory interleukins (IL)-4 and IL-6 (128).

Xanthine oxidase. Xanthine oxidase is derived from a posttranscriptional modification to the constitutively expressed xanthine dehydrogenase. Both of these enzymes oxidize hypoxanthine to xanthine and xanthine to urate. Xanthine dehydrogenase uses NAD^+ as an electron acceptor, generating NADH (a stable product). Xanthine oxidase, on the other hand, uses molecular oxygen as the electron acceptor, thereby generating $O_2^{\cdot-}$ (142, 145). Xanthine oxidase is expressed in vascular endothelial cells and CACs, and excess xanthine oxidase activity has been linked to oxidative stress-mediated dysfunction of cardiovascular tissues (130, 145, 161). It has been suggested that the primary role for endothelial cell xanthine oxidase is to recruit and activate immune cells in the inflammatory response to foreign pathogens (130, 145). These inflammatory and pro-oxidative pathways are highly activated in the injury pathogenesis of atherosclerosis (180), and xanthine oxidase activity is an important player in the oxidative stress-related development of CV disease and atherosclerosis. For example, increased xanthine oxidase activity was demonstrated to be partly responsible for hypertension and increased arteriolar tone in rats (208). Additionally, coronary artery disease patients displayed increases in xanthine oxidase activity in coronary artery endothelial cells compared to controls (202). It is important to mention that although the different sources of $O_2^{\cdot-}$ discussed in this chapter are presented and discussed in isolation, these pro-oxidant enzymes

operate in coordinated, tightly controlled networks. This is particularly evidenced by the finding that xanthine oxidase-derived $O_2^{\cdot-}$ production was completely prevented in endothelial cells lacking the NADPH oxidase subunit $p47^{phox}$ (143), suggesting that fully assembled and activated NADPH oxidase enzymatic activity is required for xanthine oxidase to produce $O_2^{\cdot-}$. Finally, all of the studies discussed above have examined xanthine oxidase in endothelial cells of the vascular system. The only study available that has examined a role for xanthine oxidase in cells with CAC functional capacity found that xanthine oxidase-derived $O_2^{\cdot-}$ stimulated proliferation of bone marrow progenitor cells (169). However, it remains to be determined whether excessive xanthine oxidase activity is involved in dysfunctional CACs in the setting of CV disease.

Mitochondrial respiration. Similar to the situations described above for $O_2^{\cdot-}$ derived from NADPH oxidase and xanthine oxidase, $O_2^{\cdot-}$ generated as a byproduct of cellular respiration acts as a signaling molecule with normal physiological functions when produced in a controlled fashion and in low amounts (45). $O_2^{\cdot-}$ is constitutively generated by complexes I and III of the mitochondrial electron transport chain (134, 154). However, here again $O_2^{\cdot-}$ in excess is associated with cellular damage resulting in negative CV health consequences. In atherosclerosis, mitochondria-derived $O_2^{\cdot-}$ production has been implicated in the processes of monocyte infiltration and their eventual transformation into lipid-laden macrophages (i.e., foam cells) (119). The accumulation of these cells at sites of endothelial damage (i.e., the atherosclerotic plaques) creates a pro-inflammatory and pro-oxidative

microenvironment. Surprisingly, no data are available to assess the role of mitochondria derived $O_2^{\cdot -}$ in CAC physiology or pathophysiology.

Antioxidant defenses. Antioxidants are defined as compounds or enzymes that are able to compete with oxidizable cellular substrates, and, thus, can prevent or delay the oxidation of those substrates (45). The enzymatic antioxidants are cytosolic SOD (SOD1), mitochondrial SOD (SOD2), catalase, and glutathione peroxidases. Both forms of SOD convert $O_2^{\cdot -}$ to hydrogen peroxide and are critical to maintaining an appropriate level of intracellular $O_2^{\cdot -}$. Catalase, which converts hydrogen peroxide to oxygen and water, and glutathione peroxidases, which prevent the oxidation of cellular macromolecules (e.g. lipids and proteins), catalyze the conversion of hydrogen peroxide (H_2O_2) generated by SOD activity to water and stable O_2 (45). Thus, adequate activities of these enzymes are required to prevent the decomposition of SOD-derived H_2O_2 to highly reactive oxygen radicals (e.g., the hydroxyl radical). CACs have been noted for their high levels of antioxidant gene expression, and this unique feature of CAC biology has been proposed as an explanation for how CACs are able to perform their reparative functions in hostile proinflammatory, pro-oxidative microenvironments [e.g., in areas of damaged endothelium or developing atherosclerotic plaque (40, 82)]. There is some, but insufficient, evidence that CAC expression and activity of antioxidant genes are responsive to prolonged oxidant exposure from the environment. For example, previous work has documented declines in intracellular antioxidant functions in CACs with age (81) and upon experimental exposure to glycation end products (30). However, whether the

antioxidant defense system is altered with lifestyle factors or disease risk factors requires additional study.

Relation of Lifestyle Factors to CAC Number and Function

Hyperglycemia and CACs. Insulin resistance and diabetes impair CAC-induced endothelial regeneration (24, 35, 112). Abnormally high blood glucose concentrations damage peripheral tissues by promoting formation of advanced glycation end products (AGEs) upon reaction of glucose and other sugars derived from glucose with components (primarily proteins) in the circulation and the intracellular environment of target tissues (68). Glycated proteins in the blood can bind to a number of AGE receptors (rAGEs) on target tissues, and rAGE signaling induces a number of oxidative stress pathways (68). In CACs, AGEs have been well documented to impair pro-angiogenic functions (30, 187, 206). In addition, excessive glucose (246) and AGEs have been shown to activate NADPH oxidase activity (177, 245). Mechanistically, $p47^{phox}$ was implicated as a factor in diabetes-related impairments in NO levels and *in vivo* regenerative capacity of CACs, as $p47^{phox}$ RNA interference restored the levels of NO and *in vivo* regenerative capacity of CACs to those of healthy controls (200). However, the role of NO depends on the source in diabetes, as iNOS-derived, but not eNOS-derived, NO was responsible for increased peroxynitrite production in a mouse model of diabetic retinopathy (50). Overall, there is sufficient evidence to conclude that a hyperglycemic environment induces substantial oxidative stress and angiogenic dysfunction in CACs.

High-fat diet and CACs. Consumption of high-fat diet is linked to a number of CV risk factors, including hyperlipidemia, hypertension, obesity, and metabolic

syndrome. Mechanistically, consumption of a high-fat diet induces oxidant stress in the vasculature and causes endothelial dysfunction (51, 65, 117). A single high-fat meal induces a severe challenge to the vascular endothelium. Flow-dependent vasodilation in large conduit vessels was decreased by ~50% at 2-4 hrs following the ingestion of a high-fat meal (237). Supplementation with dietary antioxidants in the form of vitamins (165) and phytonutrients (a mix of vegetable juice concentrate and herbal extracts) were later shown to blunt the effect of high-fat feeding on endothelial dysfunction (166), consistent with a role for oxidative stress as a mechanistic mediator of the endothelial impairment. Only one study has indirectly assessed the effect of a high-fat meal on oxidative stress and functional aspects of CACs. Liu et al. (132) investigated the effect of postprandial remnant-like particles (i.e., particles of chylomycrons and very low-density lipoproteins with known roles in vascular inflammation, oxidative stress and endothelial function) on CACs. CACs exposed to remnant lipoprotein particles exhibited impaired migratory and proliferative capacities and increased senescence compared to untreated cells. Nitrotyrosine staining was increased in remnant like particle-treated cells, and this was inhibited by pre-treatment with SOD, suggesting increased $O_2^{\cdot -}$ production and formation of peroxynitrite as a mechanism of the postprandial remnant-like particle effects on CAC function. This study indicates that remnant microparticles, which are elevated during the postprandial period following a high-fat meal, induce oxidant stress in CACs that could contribute to CV disease-associated impairments in CAC function. A limitation of this study (132) was that the remnant like particles were isolated from plasma from older hypelipidemic subjects, and the CACs were isolated from young

healthy donors. Therefore, the extent to which the results of Liu et al. can be extrapolated to the *in vivo* situation is not clear.

However, while further evidence is clearly needed with respect to CACs *per se*, there is a growing literature on the effects of a high-fat meal on lipid uptake as well as inflammatory- and oxidative stress-pathways in PBMCs (without further division into subfractions). Postprandial lipemia (PPL), i.e. the appearance of elevated blood lipids following ingestion of a meal, has been repeatedly shown to induce lipid uptake (71, 127) and activate inflammatory pathways (5, 101, 233) in mononuclear cell populations. Mechanistically, it was recently shown that a high-fat meal increases expression of the receptor for apoloprotein B-48 in circulating $CD14^+$ monocytes, which causes lipid engorgement and foam cell formation in resident macrophages (235). Monocytes also upregulate expression of CD11c/CD18, an integrin involved in monocyte adhesion to inflamed endothelium and thought to be critical in the in the accumulation of monocytes/macrophages in atherosclerotic plaques (252), in response to a high-fat meal (71). All of these studies implicated a role for inflammatory pathways in the effects of high-fat feeding on circulating monocytes; however, oxidative stress pathways have not been adequately examined. Only two studies have investigated whether oxidant status was altered in circulating cells during PPL. First, it was observed that ingestion of red wine completely prevented PPL-induced increases in nuclear factor-kappaB activation in PBMCs, an effect that was attributed to its antioxidant properties (19). Second, treatment of cells isolated in the postprandial state with the oxidative scavenger dimethylthiourea reduced the expression of neutrophil adhesion molecule CD66b by ~35% (5). Thus, there is

limited, but promising, evidence that oxidant stress pathways are at least partly responsible for dysfunctional proatherogenic effects of a high-fat meal on circulating cells. Furthermore, it can be cautiously speculated that similar effects would be observed in CACs. Given the roles of (i) CACs in maintaining vascular endothelial homeostasis, (ii) oxidative stress in CV disease- and risk factor-related CAC dysfunction, and (iii) postprandial lipemia in the development of endothelial dysfunction and atherosclerosis, it would appear that studies investigating the effects of high-fat feeding on oxidant stress in CACs are urgently needed.

Effects of acute exercise on CAC number. Acute exercise increases the number of CACs in most (124, 146, 152, 174, 193, 212, 214, 228, 231, 257) but not all (133, 212) studies. A number of factors appear to determine whether CAC numbers increase with exercise. For example, age, training status, and the nature of the exercise stimulus appear to play a role (149, 152, 212). Particularly, exercise intensity (124, 152, 231) and/or duration (124, 146) must be sufficient. In one study investigating the effects of prolonged endurance exercise (i.e., a marathon), there was no change in CACs defined as $CD34^+$/$VEGFR2^+$ cells (2). However, there was a significant decrease in $CD34^+$ progenitors, and therefore an increase in the percentage of endothelial antigen-expressing cells within the total $CD34^+$ cell pool. The authors interpreted these findings as evidence that prolonged running exercise may induce endothelial differentiation of circulating progenitors. Recently, Bonsignore et al. (21) published a comprehensive comparison of the effects of short-duration maximal exercise (a 1500m run) and prolonged endurance exercise (a marathon) on three different types of CACs ($CD34^+$/$VEGFR2^+$ EPCs, $CD133^+$/VE-cadherin$^+$ EPCs, and

cultured CACs derived from the whole PBMC pool). Levels of all three cell types increased significantly with both types of exercise. However, the magnitude of the increase in CD133^{+}/VE-cadherin^{+} EPCs was greater with marathon running compared to the 1500m run. In contrast, the number of CACs generated by the culture assay was induced to a greater extent by the 1500m run than the marathon, and the increase in CD34^{+}/VEGFR2^{+} cells did not differ between exercise bouts. In addition, the total numbers of CD34^{+} or CD133^{+} progenitor cells were unchanged with prolonged endurance exercise. Together, these data indicate that the effect of acute exercise on CAC number varies according to intensity and duration and differs among CAC subpopulations.

In terms of molecular regulation of exercise-induced CAC mobilization, the relatively rapid increase in circulating VEGF levels with acute exercise has recently been proposed to act as a stimulus for mobilization of CACs (146). Morici et al. (152) observed increases in CD34^{+} and CD133^{+} progenitors in response to supramaximal interval rowing exercise, and these changes were tightly linked with increases in levels of a number of circulating stem cell-mobilizing factors. These data suggest a possible role for cytokines in exercise-induced mobilization of progenitor cells from the bone marrow. Another important mechanism for exercise-induced CAC mobilization is NOS activity, as a recent study indicated that a significant effect of acute exercise on CAC number was completely blunted by infusion of the NOS inhibitor L-NAME (36). This finding was consistent with the previously-established requirement for eNOS in mobilization of CACs from bone marrow in a mouse model (4).

Effects of acute exercise on CAC function. Few data are available on the effects of acute exercise on functional aspects of CACs. Acute exercise reversed CAC dysfunction in heart failure patients (228), although this acute exercise effect was not observed by the same research group following a period of exercise training (230). It can be speculated that an increase in CAC intracellular NO levels could be partially responsible for the acute exercise-induced improvement in CAC, as a previous study indicated that exercise increased NO release by CACs into their culture media (257). In addition, our laboratory also recently found that acute exercise may influence the function of CACs by favorably influencing cell cycle gene expression through thrombin signaling (133), suggesting a possible role for thrombin in CAC-mediated endothelial maintenance.

Effects of chronic exercise on CAC number. The first report of exercise training-induced effects on CACs indicated that mice with access to a running wheel had higher circulating levels of spleen- and bone marrow-derived CACs than sedentary mice (125). There is evidence that oxidant stress is involved in the regulation of chronic exercise-induced changes in CAC number, as experimental knockdown of catalase activity in mice prevented increases in bone marrow CACs following a 3-wk period of physical activity (207). In humans, most investigations of the effects of exercise training on CACs have used CV disease patients as study participants. From these studies, it appears that patients with CV risk factors or overt CV disease increase CAC number with regular exercise (1, 125, 181, 203, 230). Healthy patients can also increase CAC number following exercise training (93). Cross-sectional studies of healthy subjects have been less supportive of chronic exercise effects on

baseline CAC number (133, 212, 251), although there has been one report of higher CD34$^+$ cells in trained than in sedentary older men (212). We found that the changes in CD34$^+$ and CD34$^+$/VEGFR2$^+$ cell numbers were closely related to changes in total antioxidant capacity and endothelial function during detraining in master's athletes (251), suggesting that maintenance of CAC number through regular physical activity may be an important mechanism by which regular exercise functions to preserve vascular endothelial health.

Effects of chronic exercise on CAC function. The first study investigating the effects of exercise on CACs by Laufs et al. found not only an increase in the number of CACs in both mice and humans, but also a correlation between the change in CAC number and the extent of neovascularization in the trained mice (125). Although this study is often interpreted as evidence for the involvement of CACs in training-induced angiogenesis [for example, in the recent review by Mobius-Winkler et al. (147)], the data were correlative and indirect. The best evidence supporting a role for CACs in exercise training-induced neovascularization came from a study in which mice were subjected to an experimental stroke (67), and greater numbers of genetically-labeled bone marrow cells were found in revascularized brain regions of animals with access to running wheels compared to animals who remained sedentary following stroke. Thus, bone-marrow derived CACs may play a role in exercise training-induced angiogenesis of ischemic tissues in CV disease, but clearly these data need confirmation in human subjects. In addition, while few studies have investigated endurance training effects on human CAC function, longitudinal data indicate that training can enhance migration of CACs towards angiogenic growth

factors (93). There is also evidence that proliferative capacity and/or endothelial-directed differentiation of CACs may be altered by regular exercise. Our laboratory found higher levels of endothelial gene expression in CACs from trained compared to sedentary individuals (133). Somewhat different from our finding of increased CAC differentiation towards the endothelial lineage, treatment of CACs with serum from subjects following an exercise training program significantly increased proliferative capacity of CACs (66).

Summary of Literature Review

Overall, there is sufficient evidence in the literature to state the following as facts: (i) CACs do exist and they play an important role in the maintenance of vascular endothelial integrity; (ii) NO and oxidative stress are mechanistic mediators of CAC number and function, and (iii) the number of CACs is increased by acute and chronic endurance exercise. Priorities for advancing the CAC field include the examination of whether and how acute and/or chronic exercise modifies functional aspects of CACs by altering NO- and oxidative stress-related processes. Integrative studies examining the interactive effects of physical activity and dietary factors on associated with oxidant status of CACs are especially needed. Thus, clearly much work remains for future studies to further clarify the role of CACs in acute and chronic exercise effects on vascular health.

Chapter 3: Effects of Acute and Chronic Endurance Exercise on Intracellular Nitric Oxide in Putative Endothelial Progenitor Cells: Role of NADPH Oxidase

The following is a reprint of the report of this study as it was published previously:

Jenkins et al., *American Journal of Physiology – Heart and Circulatory Physiology* 297(3): H1798-H1805, 2009.

Included with permission from the American Physiological Society (see Appendix).

Effects of acute and chronic endurance exercise on intracellular nitric oxide in putative endothelial progenitor cells: role of NADPH oxidase

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Running Head: Exercise and NO_i in putative EPCs

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ABSTRACT

We sought to delineate the effects of acute and chronic exercise on the regulation of intracellular nitric oxide (NO_i) production in putative endothelial progenitor cells (EPCs). Putative EPC colony forming units (CFU-EC) were cultured from blood drawn before and after 30 min of treadmill exercise at 75% of maximal oxygen uptake in active (n = 8) and inactive (n = 8) men. CFU-EC were similar between groups at baseline, but increased after exercise in active men only ($P = 0.04$). CFU-EC expressed lower NADPH oxidase subunit $gp91^{phox}$ mRNA and elevated endothelial nitric oxide synthase (eNOS) mRNA in active relative to inactive men at baseline ($P < 0.05$). Acute exercise reduced $gp91^{phox}$ mRNA in CFU-EC of both groups ($P < 0.05$), while $p47^{phox}$ mRNA levels were reduced in the inactive group only ($P = 0.02$). There were no differences between groups or with acute exercise in xanthine oxidase, superoxide dismutase (SOD) isoforms, or gluthathione peroxidase-1 (GPX-1) mRNA levels. NO_i was significantly greater in CFU-EC of active men at baseline ($P = 0.004$). NO_i increased in CFU-EC of inactive men with acute exercise, and *in vitro* experiments with apocynin indicated the increased NO_i production was caused by suppression of NADPH oxidase. However, the increases in NO_i with the different treatments in the inactive group did not reach the baseline levels in the active group ($P < 0.05$). We conclude that acute exercise increases NO_i in cells generated by the CFU-EC assay through an NADPH oxidase-inhibition mechanism in sedentary men. However, differences due to chronic exercise must involve additional factors. Our findings support exercise as a means to improve putative EPC function and suggest a novel mechanism that may explain this effect.

Index terms: physical activity, angiogenesis, oxidative stress

INTRODUCTION

Bone marrow-derived progenitor cells with vasculogenic capacity, often termed putative endothelial progenitor cells (EPCs), have emerged as a novel cardiovascular (CV) disease risk factor because of their role in the maintenance of vascular endothelial integrity. Circulating levels of putative EPCs independently predict CV disease progression, CV events, and endothelial dysfunction (189, 248) and EPC number and function decline with physical inactivity (211). Thus, the available data strongly implicate putative EPCs as potential targets in the primary prevention of CV disease through regular exercise.

Acute and chronic endurance exercise are thought to increase EPC number and their ability to secrete pro-angiogenic growth factors and/or incorporate into existing vascular endothelium (190, 211). A single exercise bout increases putative EPC number in humans (37), and exercise training interventions have increased putative EPC number and colony forming units (CFU-EC) in healthy subjects (93), and, most frequently, in patients with CV disease risk factors or overt CV pathologies (125, 131, 162, 203). However, while these previous studies provided strong evidence of putative EPC involvement in the exercise-induced enhancements of vascular health, the data are generally associative and mechanistic cause-effect relationships have not been established.

The signaling molecule nitric oxide (NO) plays a central role in the function of EPCs (216), as well as in mature endothelial cells (ECs) (159). Exercise training improves endothelial function by increasing vasodilatory NO release and endothelial nitric oxide synthase (eNOS) activity in ECs (125). Conversely, NO activity is

markedly reduced in the presence of CV disease risk factors including sedentary behavior (138). Physical inactivity causes endothelial dysfunction in part through impairment of eNOS (256) by upregulation of the pro-oxidant enzyme NADPH oxidase (138, 161), which generates deleterious superoxide anions and, via uncoupling of the eNOS reaction, the powerful oxidant peroxynitrite (118, 161). NADPH oxidase is one of the most important sources of oxidative stress in mature ECs throughout the CV system (159), and although the available evidence indicates that the eNOS and NADPH oxidase pathways are active in putative EPCs (48, 258), they have not been adequately characterized with respect to alteration of putative EPC function by physical activity. It is plausible that a reduction in NO generation by increased NADPH oxidase activity is a cellular mechanism for impaired putative EPC function with a sedentary lifestyle.

Therefore, the purpose of this study was to determine the effects of acute and chronic endurance exercise on the intracellular environment of putative EPCs. We hypothesized that acute exercise and regular physical activity would increase CFU-EC intracellular NO (NO_i) levels by upregulation of eNOS and suppression of NADPH oxidase. We also explored whether mRNA levels of other pro-oxidant (xanthine oxidase) and antioxidant [copper-zinc and manganese superoxide dismutases (CuZnSOD and MnSOD, respectively), and glutathione peroxidase-1 (GPX-1)] in CFU-EC were affected by acute and chronic endurance exercise.

METHODS

Screening

All participants were young, healthy, nonsmoking males with no history of CV or metabolic disease, and were not taking prescription medications. The active group (n = 8) consisted of men age 18-30 yrs with a history of >3 yrs moderate- to high-intensity endurance exercise for >4 hrs/wk. These men were recruited from local running clubs in the University of Maryland area. The inactive group (n = 8) included young men of a similar age who reported ≤20 min endurance exercise ≤2 days/wk. Groups were matched for age, body mass index (BMI), body composition, and conventional CV risk factor profile. All participants provided written informed consent prior to all testing and the University of Maryland Institutional Review Board approved all study procedures.

Maximal graded exercise test and body composition

All testing occurred in the morning after an overnight fast and refraining from alcohol, vitamins, and caffeine for 24 hrs. Height, weight, and blood pressure were measured, and body fatness was estimated using the seven-site skinfold procedure (107). Maximal oxygen uptake ($\dot{V}O_2max$) was assessed using a constant-speed treadmill protocol with 2% increases in incline every 2 min until exhaustion. The treadmill speed was chosen by the investigators based on subject experience, typical running speed, and heart rate such that $\dot{V}O_2max$ was achieved in ~6-12 min. Expired gases were analyzed using an automated indirect calorimetry system (Oxycon Pro, Cardinal Health, Inc.; Dublin, OH). $\dot{V}O_2$ was considered maximal using the plateau criteria, and all tests met at least two of the following secondary criteria of maximal effort: a respiratory exchange ratio of >1.10, a rating of perceived exertion of ≥19, and/or a peak heart rate within 10 beats per minute of the age-predicted maximum

(6). Heart rate was measured during testing using heart rate monitors (Polar Electro, Inc.; Woodbury, NY).

Blood sampling and steady state exercise test

Participants reported to the laboratory after an overnight fast for experimental testing 48-72 hrs after completing the $\dot{V}O_2max$ and body composition assessments. A blood sample for baseline CFU-EC and standard CV risk factor assessments was drawn immediately before exercise, and a second sample was obtained for CFU-EC 30 min after completing a 30-min treadmill run at 75-80% $\dot{V}O_2max$. Treadmill running speed was the same as that used for the $\dot{V}O_2max$ test and the appropriate percent incline was estimated from the American College of Sports Medicine equation for $\dot{V}O_2$ during treadmill running (6). Intensity during exercise was monitored using the heart rate reserve method.

Colony forming unit-endothelial cell (CFU-EC) assay

The CFU-EC assay was performed as described previously (91). Briefly, mononuclear cells were isolated from peripheral blood samples obtained before and 30 min after exercise by density gradient centrifugation (Ficoll Paque Plus, GE Healthcare; United Kingdom). The cells were washed twice with PBS supplemented with 2% FBS, and plated at 5×10^6 cells/well on 6-well culture plates coated with human fibronectin (BD Pharmingen; Franklin Lakes, NJ) in 2 mL Endocult Medium (Stem Cell Technologies; Vancouver, BC). Non-adherent cells were harvested after a 48-hr incubation in a humidified incubator (37°C, 5% CO_2) and replated (1×10^6 cells/well) on 24-well fibronectin-coated plates (BD-Pharmingen) in 1 ml Endocult Medium. CFU-EC appeared 3d later and were defined according to previously

established methodology which includes central cores of round cells with more elongated sprouting cells at the periphery (91). The endothelial lineage of these cells has been confirmed previously by immunocytochemical staining for von Willebrand factor, vascular endothelial growth factor receptor-2, and CD31 (91). Investigators trained in identification of colonies but blind to the status of the sample performed CFU-EC counts in four randomly chosen wells. The correlation between observers in our laboratory for CFU-EC counts was 0.98 ($p < 0.001$). To reduce assay variability, all experiments in this study were performed with the same stock and lot of Endocult basal medium and supplements.

Gene expression by semiquantitative RT-PCR

eNOS, NADPH oxidase (subunits gp91^{phox}, p47^{phox}, and p67^{phox}), xanthine oxidase, CuZnSOD, MnSOD, and GPX-1 mRNA levels were measured using semiquantitative RT-PCR. Total RNA was extracted in quadruplicate from CFU-EC cultured for 5 days using the TRI reagent (Sigma-Aldrich; St. Louis, MO) according to previously described methods (201). RNA quantity was calculated from absorbance at 260 nm and quality was verified by the 260:280 absorbance ratio. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc.; Carlsbad, CA) according to the manufacturer's instructions. cDNA was amplified using specific primers under optimal thermal cycling conditions determined empirically for each target gene as previously described (201). The primer sequences and thermal cycling conditions are presented in Table 2. PCR products were separated by agarose gel electrophoresis and photographed under ultraviolet light. Signal intensities were semi-quantified

using NIH imaging software (ImageJ) and normalized to the signal for the reference gene 18S.

Experimental blockade of NADPH oxidase activity

NADPH oxidase activity in CFU-EC was inhibited using the drug apocynin (Sigma-Aldrich) in *ex vivo* experiments to determine if exercise increases CFU-EC NO_i via an NADPH oxidase-dependent mechanism. Apocynin prevents assembly of the enzyme by reacting with thiol groups required for the translocation of the intracellular subunits $p47^{phox}$ and $p67^{phox}$ to the catalytic $gp91^{phox}$ subunit, in turn preventing the generation of deleterious superoxide radicals (204). Cells of active and inactive groups from before and after acute exercise were incubated from day 4 to day 5 of the CFU-EC assay with 100 μM apocynin in 1 ml Endocult medium. Cells treated with the same volume of the apocynin vehicle (10 μl PBS) in 1 ml Endocult medium were used as a control. The apocynin concentration and the 24-hr treatment were empirically determined to produce detectable changes in NO_i. We aimed to not affect the early events of the assay such as adherence of the cells to the plate or initial differentiation into CFUs, but we did want to allow sufficient time for any measurable effects of apocynin on NO_i to appear. An apocynin-induced increase in endothelium-dependent dilation and its NO component in mouse aorta was recently observed in as little as 60 min (46). Therefore, we believe the 24-hr experiment was valid for our purposes. The experiment consisted of the following conditions for both active and inactive group CFU-EC: baseline-vehicle (BL-VEH), baseline-apocynin (BL-APO), exercise-vehicle (EX-VEH), and exercise-apocynin (EX-APO). These experiments

were performed on CFU-EC from a subset of individuals (n = 3-5 per group per condition).

Detection of intracellular nitric oxide in CFU-EC

NO_i was measured using the DAF-FM diacetate fluorescent dye technique (Molecular Probes, Carlsbad, CA) as described by Drenning et al. (44), with minor modifications. DAF-FM diacetate is a pH-insensitive dye that emits fluorescence on reacting with an intermediate of NO during the spontaneous oxidation of NO to NO_2^-. On day 5 of the CFU-EC assay, media were removed, CFU-EC in triplicate wells were washed twice with 500 µl PBS, loaded with 500 µl PBS containing 10 µM DAF-FM diacetate, and incubated for 30 min at 37°C. Dyed cells were washed twice with PBS, and NO_i fluorescence was measured using a multi-label plate reader (Wallac 1400 VICTOR^2, Perkin Elmer, Inc., Waltham, MA) with excitation and emission wavelengths of 488 and 535 nm, respectively. Every CFU-EC plate included the following controls: (i) duplicate wells of unloaded cells to serve as a control for cellular autofluorescence, (ii) duplicate wells that contained no cells, but contained 10 µM DAF-FM in PBS to correct for any fluorescence resulting from the dye itself, and (iii) duplicate wells of 500 µl PBS alone. As the arbitrary-type fluorescence among these three control conditions were similar in pilot experiments ($P = 0.7$, data not shown), the average fluorescence value of the three controls was subtracted from each experimental value. The average within- and between-assay coefficients of variation for the arbitrary fluorescence were 5% and 9%, respectively, indicating good agreement in arbitrary fluorescence between wells on the same plate

and among experiments conducted on different days. Data are expressed as fold difference from the mean for the active group's CFU-EC in the BL-VEH condition.

In addition, we must point out that gene expression and NO_i measurements were made on all cells present in the CFU-EC assay, and not the colonies alone. Thus, the term "CFU-EC" must be interpreted to mean all cells cultured in the CFU-EC assay when we refer to our NO_i and gene expression data.

Statistics

Between-group and acute exercise effects were analyzed by independent and paired samples t-tests, respectively, according to *a priori* planned contrasts. Within- and between-group ANOVA with Dunnett’s post hoc tests were used to determine whether exercise and/or apocynin treatments increased NO_i relative to BL-VEH. We used the $\alpha = 0.05$ criterion for statistical significance. Data are presented as mean ± SE unless indicated otherwise.

RESULTS

Active and inactive participants were successfully matched on the basis of age, BMI, and the standard CV risk factor profile, but differed significantly in terms of $\dot{V}O_2max$ (Table 1).

CFU-EC counts

CFU-EC counts were not different between groups at baseline ($P = 0.23$; Figure 3.1A). CFU-EC increased after exercise in the active group ($P = 0.02$), but did not change in the inactive group ($P = 0.6$; Figure 3.1B).

Gene Expression

eNOS mRNA levels were elevated in CFU-EC from active relative to inactive men by ~30% at baseline ($P = 0.04$) and by ~17% after exercise ($P = 0.05$), but did not change with exercise in either group ($P \approx 0.4$ for exercise-induced changes in both groups) (Figure 3.2). $gp91^{phox}$ mRNA levels were ~44% lower in CFU-EC from active than inactive men at baseline ($P = 0.02$) and were reduced after acute exercise in both groups (active, $P = 0.02$; inactive, $P = 0.04$), with expression remaining significantly ($P = 0.01$) higher after exercise in CFU-EC of inactive compared to active men (Figure 3.3A). $p47^{phox}$ mRNA levels did not differ between groups at baseline but decreased by ~20% after exercise in the inactive group ($P = 0.02$) (Figure 3.3B). There were no differences between groups or with acute exercise in $p67^{phox}$ mRNA levels (Figure 3.3C) or xanthine oxidase (Figure 3D) mRNA levels ($P > 0.05$). There were also no differences between groups or with acute exercise in expression of the antioxidant genes CuZnSOD, MnSOD, or GPX-1 (Figure 3.4A-C; $P > 0.05$).

Intracellular nitric oxide (NO_i)

Detectable NO_i was ~56% greater in the cells cultured in the CFU-EC assay from active compared to inactive men ($p = 0.004$) (Figure 3.5). In the active group there were no significant differences among BL-VEH, BL-APO, EX-VEH, or EX-APO in CFU-EC NO_i (ANOVA $P > 0.05$). In the inactive group, BL-APO ($P = 0.04$), EX-VEH ($P = 0.04$), and EX-APO ($P = 0.02$) all significantly increased NO_i levels relative to the BL-VEH condition. However, there were no differences in NO_i among these three experimental conditions for the inactive group (all $P > 0.05$). Additionally, inactive group NO_i levels were significantly lower compared to the

active group BL-VEH in all experimental conditions (BL-APO, EX-VEH, and EX-APO; all $P < 0.05$). Colony formation was not affected by VEH or APO treatments (data not shown); therefore, CFU-EC count data are from VEH or untreated samples.

DISCUSSION

The main findings of this study are (i) acute exercise increases CFU-EC NO_i levels in sedentary individuals, (ii) regular endurance exercise is associated with increased eNOS gene expression and NO_i in CFU-EC, (iii) the acute exercise effect on NO_i is NADPH oxidase-dependent, and (iv) the training effect on NO_i appears to involve other mechanisms besides reduced NADPH oxidase activity. Importantly, these differences were observed between groups of healthy young men who were matched for age, BMI, and the standard CV risk factor profile, and differed only in $\dot{V}O_2max$. Our findings support the notion that acute and chronic exercise improve putative EPC function and suggest a novel cellular mechanism that may explain this effect.

NADPH oxidase is regarded as one of the most important sources of oxidative stress in the CV system (78, 159, 161). ECs throughout the CV system express elevated NADPH oxidase in several pathological conditions associated with physical inactivity, including CV disease (3), hypertension (111), and diabetes (155). In putative EPCs, excessive NADPH oxidase-derived superoxide radicals promote premature cellular senescence and reduced proliferative capacity (103). Here, we show elevated expression of the NADPH oxidase catalytic subunit $gp91^{phox}$ gene in CFU-EC of healthy men who would be considered very healthy, and have excellent CV disease risk profiles, but who do not regularly perform endurance exercise. We

show further that a single bout of exercise reduces mRNA expression of $gp91^{phox}$ and $p47^{phox}$ in CFU-EC NO_i from these men. To our knowledge, no studies have assessed the effects of acute or chronic endurance exercise on putative EPC NADPH oxidase gene expression, but these data are consistent with a previous report of CV disease patients showing a reduction in $gp91^{phox}$ mRNA in coronary artery ECs following a 4-wk endurance training program (3).

Because eNOS uncoupling, and subsequent depletion of NO_i, is a consequence of elevated NADPH oxidase activity (118, 161), we tested whether the observed differences in eNOS and NADPH oxidase gene expression between groups and with acute exercise were corroborated by differences in CFU-EC NO_i. Consistent with the elevated baseline $gp91^{phox}$ and reduced eNOS mRNA levels in the inactive group, these individuals also displayed significantly lower NO_i compared to the active group. The inactive group NO_i levels increased with BL-APO, EX-VEH, and EX-APO treatments. Importantly, the effects of NADPH oxidase inhibition and acute exercise on NO_i were nearly identical in magnitude and were not additive, as evidenced by no further increase in NO_i in the EX-APO condition over either treatment alone. Further, there was no acute exercise effect on eNOS mRNA in either group, despite a between-group difference at baseline. Together, these data indicate that in inactive individuals, NO_i in putative EPCs is increased by acute endurance exercise through an NADPH oxidase-driven mechanism. This is a critical finding of the study, but this mechanism only partly explains the training-related differences in NO_i in cells of the CFU-EC assay. The increases in the inactive group's NO_i by apocynin, exercise, and their combination were not sufficient to reach the levels of the active group's CFU-EC

NO_i under the BL-VEH condition. Thus, the exercise training-induced difference in NO_i is also likely explainable by the elevated baseline eNOS gene expression in the active group as well as other mechanisms independent from NADPH oxidase.

The changes observed with acute exercise in the inactive group were not observed in the active group. CFU-EC of these individuals displayed lower $gp91^{phox}$ mRNA at baseline and decreased expression of $gp91^{phox}$ following exercise, but this change was apparently without consequence for NO_i levels, which remained higher in CFU-EC from active men under all experimental conditions. It is reasonable to speculate that we have observed a ceiling effect for the active individuals' NO_i levels.

Most reports of acute exercise and exercise training effects on putative EPCs have enumerated cells by colony-forming potential in culture or flow cytometry. In general, exercise training increases putative EPC number and colony-forming potential (93, 125, 162, 203), but this has not always been the case (212). The available data are also equivocal as to whether an acute exercise bout increases putative EPCs, with some studies showing increased EPC number (124, 231) and colony formation (70, 124, 174) after acute exercise, but others showing no effect on putative EPC number (212) or colony formation (231). The most consistent effects of acute and chronic exercise have been observed in patient populations with overt endothelial dysfunction and CV pathologies (125, 162, 203). Of all these studies, the acute exercise CFU-EC data in the present study are most consistent with (i) a report in which CFU-EC increased in endurance-trained individuals after exercise of equivalent intensity and duration to that in the present study (30 min at ~80% $\dot{V}O_2max$) (124), and (ii) another report showing no change in CFU-EC after exercise in

a group of untrained, but healthy, men and women (231). Therefore, taken with previous data, the present study suggests a relationship between participant fitness and the response of putative EPC colony formation to acute exercise, but this hypothesis needs further attention.

However, the effects of exercise on the EPC intracellular environment may well be far more important than acute or chronic exercise effects on putative EPC number or colony formation. From our data, a reasonable working hypothesis is that the intracellular environment of a putative EPC may influence its functional capacity and its ability to affect endothelial function. NADPH oxidase and eNOS were chosen as target genes in this study because of their well-characterized role in the (dys)function of the endothelium throughout the CV system. Clearly, our evidence suggests that NO_i production is altered in putative EPCs due to changes in activity, indicating an important effect of exercise on the EPC intracellular environment. Our evidence further suggests that an impaired intracellular redox state resulting from high NADPH oxidase activity may be detrimental to putative EPC function in young, healthy men with a sedentary lifestyle.

Our findings indicate a role for NADPH oxidase in the regulation of NO_i in cells generated by the CFU-EC assay. However, additional factors were implicated in training-related differences as well as the response to acute exercise in inactive subjects. Thus, we also investigated the expression of other genes involved in intracellular redox status. The findings that mRNA levels of antioxidant (CuZnSOD, MnSOD, and GPX-1) and pro-oxidant (xanthine oxidase) genes did not differ between groups or with acute exercise support the role for NADPH oxidase as a key

mediator of NO_i dynamics. However, these data do not clarify the complete training-related or acute exercise-induced differences in NO_i. Further work will be necessary to fully identify all the factors explaining the differences NO_i regulation in putative EPCs between the trained vs. untrained states. We speculate that there may be differences in the activities or responsiveness of these or other oxidases or antioxidant systems that we did not detect at the mRNA level.

We must acknowledge three limitations of our study. First, NO_i and gene expression were measured from all cells present in the dish, and not the colonies themselves. Fluorescence microscopy experiments have documented eNOS activity in cells generated by the CFU-EC assay (158), but the amount of NO release by the colonies relative to the other cells present in the dish has not been determined. Therefore, it is unclear if differences detected in NO_i measures were due to changes in NO production in the colonies themselves, or due to changes in a subpopulation of cells outside the colonies that express eNOS and/or NADPH oxidase. However, the data of Hill and colleagues (91) suggest that it is the CFU-EC themselves that express endothelial markers, and the colonies have been repeatedly shown to take up acetylated LDL and bind to lectin, suggesting an endothelial phenotype. Second, there are conflicting reports about the identity of cells generated by the CFU-EC assay. An emerging hypothesis suggests that CFU-EC may be T cells (41) with a distinct angiogenic phenotype (100). There are also very recent data on the existence of a novel population of circulating CD31+ T cells that secrete angiogenic growth factors (121), which is one mechanism by which CFU-EC have been proposed to promote new vessel growth (100, 224). We believe our data provide new information

on how acute and chronic endurance exercise may alter the function of these cells, but we must emphasize that the precise identities of these cells and other putative vasculogenic progenitors are still being elucidated. Finally, while it appears likely that the effect of acute exercise on NO_i was NADPH oxidase-dependent based on our study design, we must be somewhat circumspect with this conclusion. We acknowledge that a number of other factors affect NO throughout the CV system (159).

In conclusion, we found a cross-sectional difference between active and inactive young men in NO_i in cells cultured in the CFU-EC assay that may be partly explained by elevated NADPH oxidase with physical inactivity. Acute exercise appears to attenuate some, but not all, of this difference through suppression of NADPH oxidase activity, indicating the existence of a training effect that must be due to elevated eNOS and other factors. Our findings may have important implications for the role of putative EPCs in the maintenance of a healthy endothelium through physical activity.

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Table 3.1. Reverse transcriptase-polymerase chain reaction procedures.

Target	Oligo Sequence	PCR Conditions	Cycles (n)	Amplicon Length (bp)
eNOS	F: 5'-ATGAAGCACCTGGAGAATGAG-3' R: 5'-TCGGAGCCATACAGGATTG-3'	95°C for 30s, 55°C for 30s, 72°C for 40s	33	299
gp91phox	F: 5'-CAACAAGAGTTCGAAGACAA-3' R: 5'-CCCCTTCTTCTTCATCTGTA-3'	95°C for 30s, 59°C for 30s, 72°C for 30s	35	689
p47phox	F: 5'-CACGGACAACCAGACAAAAA-3' R: 5'-AGAACCACCAACCGCTCTC-3'	95°C for 30s, 59°C for 30s, 72°C for 30s	24	203
p67phox	F: 5'-TGGAGGAGTTAGGGGAGAGG-3' R: 5'-CCTGGACTTGGGTGTCTTGT-3'	95°C for 30s, 50°C for 30s, 72°C for 30s	26	219
XO	F: 5'-CTTGAAAGGCTGAGGTGGAG-3' R: 5'-GGGGAATTGACAGTCCAAGA-3'	95°C for 30s, 61.5°C for 30s, 72°C for 30s	30	248
CuZnSOD	F: 5'-ATGACTTGGGCAAAGGTGGAAATG-3' R: 5'-GTTAAGGGGCCTCAGACTACATCC -3'	95°C for 30s, 54.5°C for 30s, 72°C for 30s	30	126
MnSOD	F: 5'-TTGGCCAAGGGAGATGTTAC-3' R: 5'-AGTCACGTTTGATGGCTTCC-3'	95°C for 30s, 53°C for 30s, 72°C for 30s	35	157
GPX-1	F: 5'-CCAGTCGGTGTATGCCTTCT-3' F: 5'-GCTGCAGCTCGTTCATCTG-3'	95°C for 30s, 53°C for 30s, 72°C for 30s	30	152
18S	F: 5'-TTGATTAAGTCCCTGCCCTTTGT-3' R: 5'-CGATCCGAGGGCCTAACTA-3'	-	-	80

Abbreviations: eNOS, endothelial nitric oxide synthase; XO, xanthine oxidase; SOD, superoxide dismutase; GPX, glutathion peroxidase.

Table 3.2. Participant characteristics. Data are mean ± S.D.*

	Active (n = 8)	**Inactive** (n = 8)	$\boldsymbol{P}$
Age (y)	25 ± 4	25 ± 3	0.82
Height (m)	1.81 ± 0.1	1.81 ± 0.04	0.96
Weight (kg)	78.8 ± 13.2	77.9 ± 17.2	0.90
BMI ($kg \cdot m^{-2}$)	24.0 ± 3.8	23.6 ± 4.4	0.86
Fat (%)	14.1 ± 5.4	14.8 ± 6.7	0.82
FFM (kg)	67.3 ± 8.8	65.4 ± 9.6	0.70
Glucose ($mg \cdot dl^{-1}$)	84 ± 8	81 ± 8	0.45
TC ($mg \cdot dl^{-1}$)	149 ± 21	147 ± 25	0.87
HDL ($mg \cdot dl^{-1}$)	53 ± 5	49 ± 11	0.31
LDL ($mg \cdot dl^{-1}$)	81 ± 21	81 ± 22	0.99
TG ($mg \cdot dl^{-1}$)	70 ± 18	82 ± 30	0.40
SBP (mm Hg)	118 ± 6	121 ± 5	0.29
DBP (mm Hg)	75 ± 10	79 ± 6	0.39
$\dot{V}O_2max$ ($L \cdot min^{-1}$)	4.7 ± 0.6	3.6 ± 0.4	**0.001**
$\dot{V}O_2max$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	60.2 ± 5.4	47.3 ± 5.7	$\boldsymbol{P < 0.001}$
$\dot{V}O_2max$ ($ml \cdot kg\ FFM^{-1} \cdot min^{-1}$)	70.1 ± 5.1	55.5 ± 4.3	$\boldsymbol{P < 0.001}$

**Abbreviations:* BMI, body mass index; FFM, fat-free mass; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure; $\dot{V}O_2max$, maximal oxygen uptake.

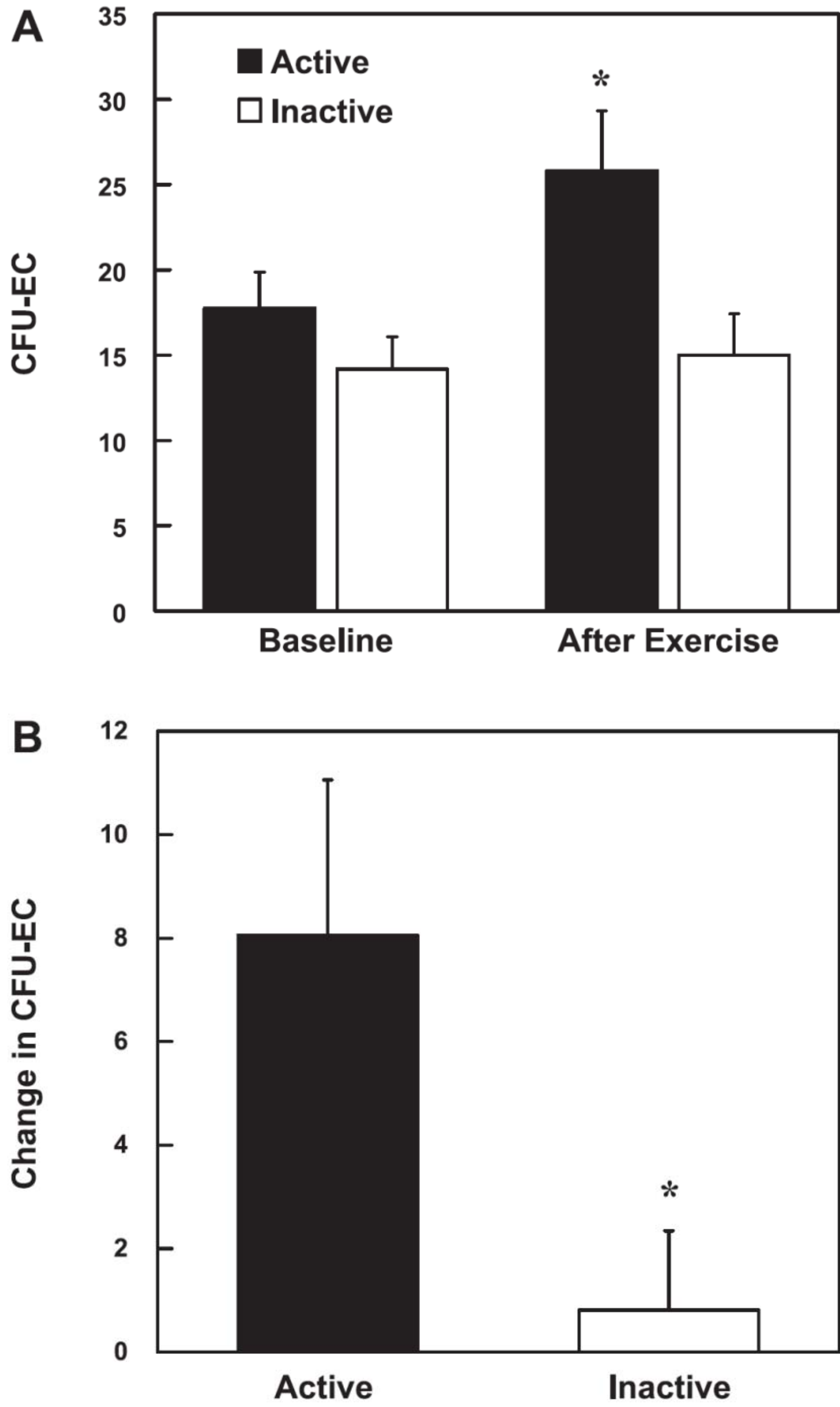

Figure 3.1. (A) Endothelial cell colony forming units (CFU-EC) in active and inactive men at baseline and after 30 min exercise at 75% $\dot{V}O_2$max. *Significantly different from baseline value ($P < 0.05$). (B) Acute exercise-induced change in CFU-EC in active and inactive men. *Significantly smaller change in inactive than active men ($P < 0.05$).

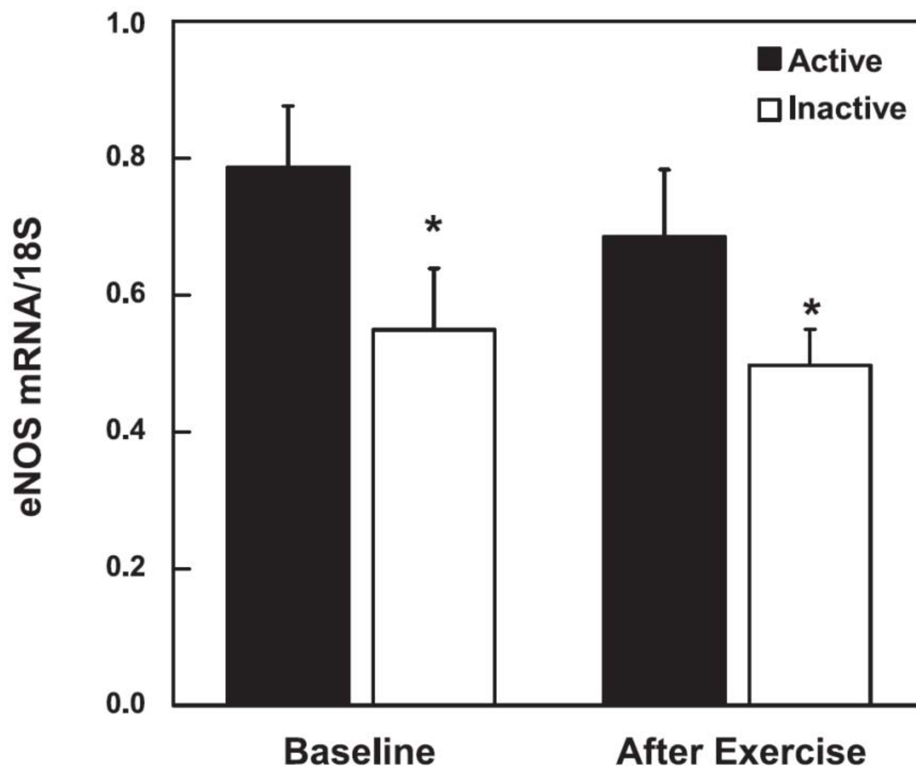

Figure 3.2. eNOS mRNA as measured by semiquantitative reverse transcriptase-PCR in active and inactive men. *Significant difference between groups ($P < 0.05$).

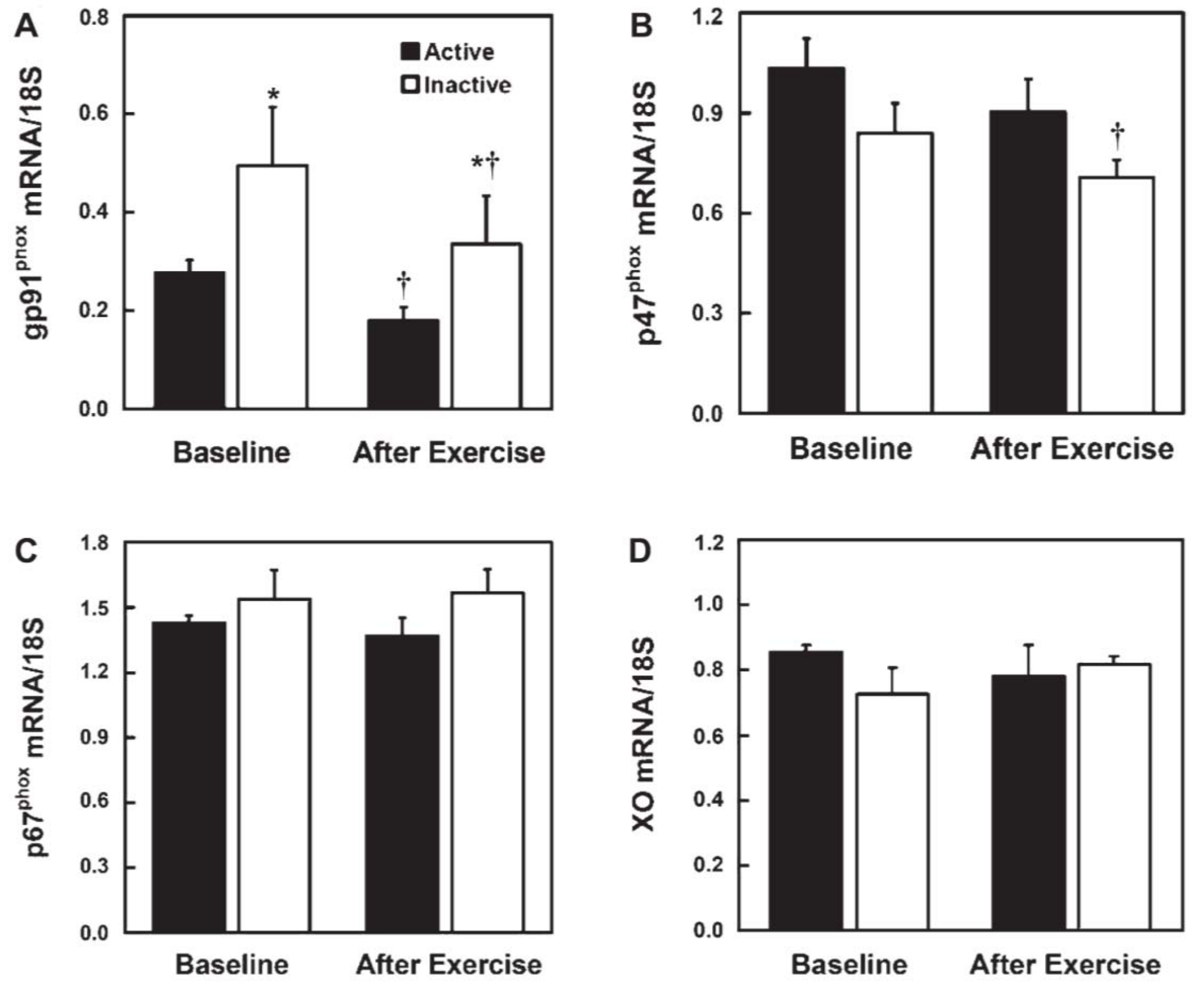

Figure 3.3. mRNA levels of the NADPH oxidase subunits gp91^{phox} (A), p47^{phox} (B), and p67^{phox} (C), and xanthine oxidase (XO). *Significant difference between groups ($P < 0.05$); †Significant within-group difference (after vs. before acute exercise; $P < 0.05$).

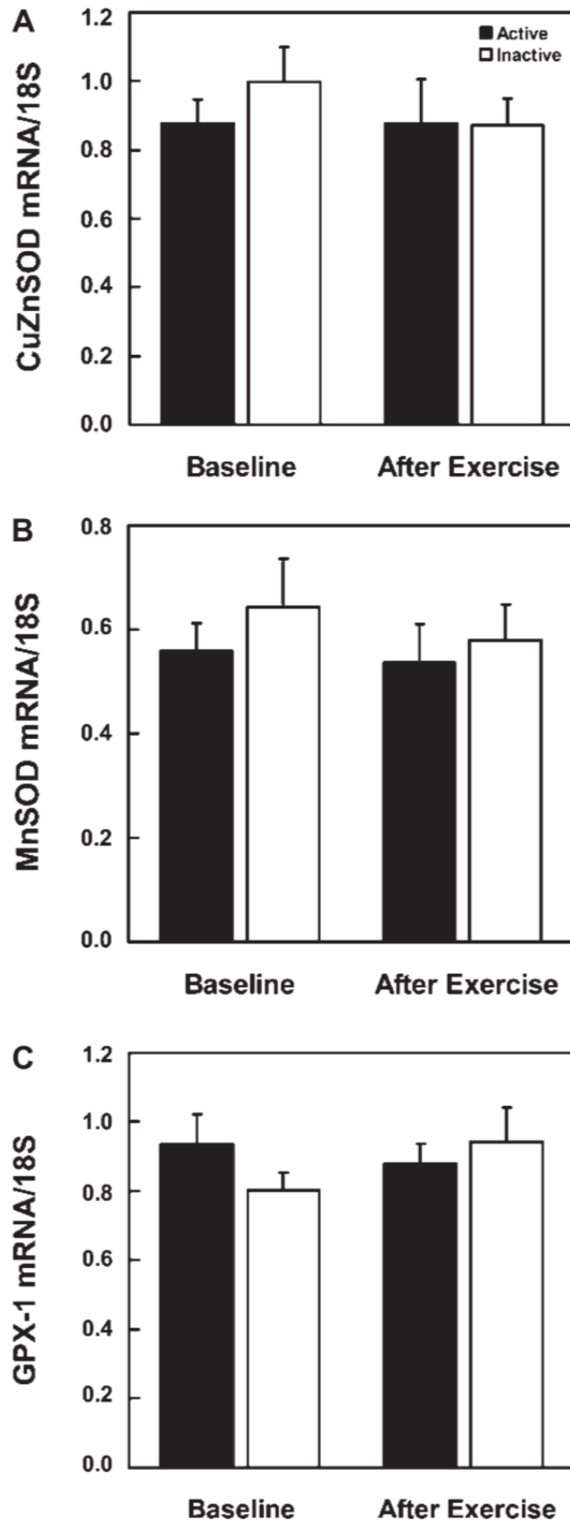

Figure 3.4. mRNA levels of CuZnSOD (A), MnSOD (B), and GPX-1 (C). There were no significant differences between groups or with acute exercise in either group ($P > 0.05$).

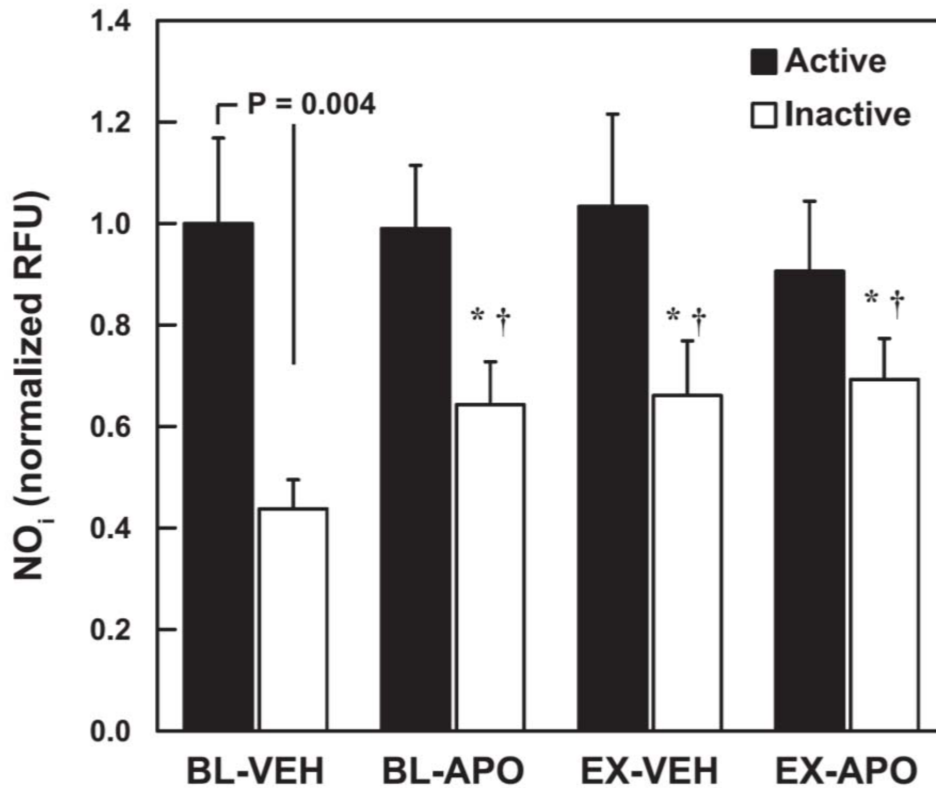

Figure 3.5. Effects of acute exercise and NADPH oxidase inhibition on intracellular nitric oxide (NO_i) in CFU-EC from active and inactive men. Relative fluorescence units (RFU) were normalized to the mean for the active group baseline-vehicle (BL-VEH) condition. *Significant difference from within-group BL-VEH condition ($P < 0.05$). †Significantly different from active group CFU-EC under the BL-VEH condition ($P < 0.05$).

Chapter 4: Effects of Acute and Chronic Endurance Exercise on Intracellular Nitric Oxide and Superoxide in Circulating CD34+ and CD34- Cells

The following is a reprint of the report of this study as it will be published in the *Journal of Applied Physiology* (Epub June 23, 2011). Permission from the American Physiological Society to include this manuscript in this dissertation is pending.

Effects of acute and chronic endurance exercise on intracellular nitric oxide and superoxide in circulating CD34$^+$ and CD34$^-$ cells

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Running Title: NO and $O_2^{\cdot -}$ in CD34$^+$ PBMCs

ABSTRACT

We investigated the influence of acute and chronic endurance exercise on levels of intracellular nitric oxide (NO), superoxide ($O_2^{\cdot -}$), and expression of genes regulating the balance between these free radicals in $CD34^+$ and $CD34^-$ peripheral blood mononuclear cells (PBMCs; isolated by immunomagnetic cell separation). Blood samples were obtained from age- and body mass index (BMI)-matched endurance-trained (n = 10) and sedentary (n = 10) men before and after 30 min of exercise at 75% maximal oxygen uptake ($\dot{V}O_2max$). Baseline levels of intracellular NO (measured by DAF-FM diacetate) and $O_2^{\cdot -}$ (measured by dihydroethidium) were 26% ($P < 0.05$) and 10% ($P < 0.05$) higher, respectively, in $CD34^+$ PBMCs from the sedentary group compared to the endurance-trained group. $CD34^+$ PBMCs from the sedentary group at baseline had 2-fold greater inducible nitric oxide synthase (iNOS) mRNA and 50% lower endothelial NOS (eNOS) mRNA levels compared to the trained group ($P < 0.05$). The baseline group difference in $O_2^{\cdot -}$ was eliminated by acute exercise. Experiments with apocynin indicated that the training-related difference in $O_2^{\cdot -}$ levels was explained by increased NADPH oxidase activity in the sedentary state. mRNA levels of additional angiogenic and antioxidant genes were consistent with a more angiogenic profile in $CD34^+$ cells of trained subjects. $CD34^-$ PBMCs, examined for exploratory purposes, also displayed a more angiogenic mRNA profile in trained subjects, with vascular endothelial growth factor (VEGF) and eNOS being more highly expressed in trained subjects. Overall, our data suggest an association between the sedentary state and increased nitro-oxidative stress in $CD34^+$ cells.

Keywords: stem cells, cardiovascular disease, physical activity

INTRODUCTION

CD34$^+$ peripheral blood mononuclear cells (PBMCs) are stem/progenitor cell precursors for hematopoietic and endothelial cells (11). Given appropriate environmental cues, CD34$^+$ PBMCS can perform beneficial angiogenic functions (11, 42) and subsets of CD34$^+$ cells co-expressing endothelial antigens have endothelial progenitor cell (EPC) characteristics. However, controversy over precise definitions of EPCs and other angiogenic cells has slowed progress in the field (53, 92), making it difficult to reach consensus on which cells should be targeted for investigation of exercise-induced effects on cell-based endothelial repair mechanisms. It is generally agreed upon that CD34$^+$ progenitor cells, whether through actions of particular endothelial-directed subsets or by acting as a pool of generic progenitors, perform proangiogenic actions that contribute to the maintenance of vascular endothelial integrity (55, 56). CD34$^+$ cell numbers predict cardiovascular (CV) disease risk and are functionally susceptible to adverse effects resulting from lifestyle related metabolic and CV disease *in vivo* environment (52, 55, 56). In addition, CD34$^+$ PBMCs have recently received a great deal of attention in the literature for cell therapy applications, with a recent study finding a reduction in myocardial infarct size following coronary infusion of CD34$^+$ bone marrow cells (171). However, no studies have investigated the effects of acute or chronic exercise on functional aspects of CD34+ cells.

There is growing evidence that acute and chronic endurance exercise increase the functional capacity of circulating angiogenic cells (93, 109, 198, 228-230), but the mechanisms underlying the functional adaptations to exercise training are not clear.

The balance between nitric oxide (NO) and superoxide ($O_2^{\cdot -}$) production is a critical mechanistic aspect of the angiogenic functions of cultured progenitor cells (61, 216), and there is a link between CV/metabolic disease and reduced NO/increased $O_2^{\cdot -}$ production in EPCs (199, 215). There have been some recent investigations into whether exercise training favorably alters the balance between NO and $O_2^{\cdot -}$ levels in circulating angiogenic cells. For example, we reported that increased activity of the pro-oxidant enzyme NADPH oxidase is a mechanistic mediator of lower intracellular NO levels in cultured EPCs of sedentary compared to trained men (109). Additionally, a recent study found that exercise training in metabolic syndrome patients increases NO production and reduced $O_2^{\cdot -}$ production in cultured EPCs, in conjunction with increased endothelial repair capacity in vivo (199). However, the effects of exercise training on intracellular NO and $O_2^{\cdot -}$ need to be examined in CD34+ cells, given their angiogenic properties discussed above. Furthermore, no exercise-based EPC studies have tested the assumption that the phenotype of cells generated after several days in culture under powerful angiogenic conditions accurately reflects the in vivo phenotype, and thus there is a need to examine cells in their freshly-isolated state.

Another issue that has not received adequate attention is whether acute exercise alters oxidant status of circulating angiogenic cell types. Acute exercise increases NO production (109) and the migratory capacity (228) of cultured EPCs. However, no studies have directly assessed the effects of acute exercise on $O_2^{\cdot -}$ production in any angiogenic cell type. Given the known role for acute exercise-induced oxidative stress as a stimulus for beneficial adaptive responses in the form of increased NO

bioavailability and up-regulation of antioxidant defense systems in muscle (110) and the vasculature (69), there is a need to investigate the effects of acute exercise on circulating cells with angiogenic function.

Therefore, we tested the hypothesis that acute and chronic endurance exercise would favorably influence the balance between NO and $O_2^{\cdot -}$ in freshly-isolated $CD34^+$ PBMCs, and elevated NADPH oxidase activity in cells of the sedentary group would be a causal mechanism underlying these effects. We also hypothesized that the expression of genes involved in the regulation of intracellular NO and $O_2^{\cdot -}$ levels would be consistent with greater antioxidant capacity and angiogenic function in the trained state. Finally, although there are angiogenic monocytic and T-cell subpopulations within the CD34- PBMC fraction (15, 34, 79, 120, 122, 240), no studies have examined the effects of acute or chronic endurance exercise on their functional properties. Thus, we also explored the effects of acute and chronic exercise on NO, $O_2^{\cdot -}$, and gene expression in $CD34^-$ PBMCs.

METHODS

Screening. All potential participants completed medical and physical activity history questionnaires that we have used previously (109). All participants were nonsmoking men age 18-35 with no history and currently free of CV disease and diabetes. Subjects were normotensive and were not on cholesterol, antihypertensive, or antihyperglycemic agents. Endurance-trained individuals (n = 10) performed at least 4 hr/wk of endurance exercise, and sedentary individuals (n = 10) reported engaging in exercise for <20 min/d on <2 days/wk. Groups were matched for age and body mass index. The study was approved by the University of Maryland College

Park Institutional Review Board and all participants provided written informed consent.

Maximal graded exercise test and body composition. All testing occurred in the morning after an overnight fast. Participants refrained from alcohol, vitamins, and caffeine for 12 hrs and antihistamines or NSAIDs for 24 hrs prior to testing. Body composition was estimated using the 7-site skinfold procedure (107). Maximal oxygen uptake ($\dot{V}O_2max$) was assessed using a constant-speed treadmill protocol with 2-3% increases in incline every 2 min until exhaustion. Subjects ran at a treadmill speed chosen by the investigator based on subject experience, typical run speed, and heart rate such that $\dot{V}O_2max$ was achieved in 6-12 min. Pulmonary ventilation and expired gas concentrations were analyzed in real time using an automated computerized indirect calorimetry system (Oxycon Pro, Viasys). $\dot{V}O_2$ was considered maximum if a plateau was achieved (increase in $\dot{V}O_2$ of < 250 ml/min with increased work). In the absence of a clear plateau, tests were verified to meet at least two of the following secondary criteria of maximal effort: a respiratory exchange ratio >1.10, a rating of perceived exertion >18, and a peak heart rate within 10 beats/min of the age-predicted maximum. Heart rate was measured during the test using heart rate monitors (Polar).

Submaximal exercise test. Subjects reported to the laboratory 2-7 days following their $\dot{V}O_2max$ test. The endurance-trained subjects performed one of their usual exercise training sessions 16-24 hrs before this test. Seated blood pressure was measured and blood samples were obtained before and after exercise for assessment of conventional CV risk factors (baseline sample only), hematocrit, hemoglobin, and

isolation of circulating $CD34^+$ and $CD34^-$ cells. The exercise consisted of 30 min of treadmill running at 75% of the subject's $\dot{V}O_2max$. The treadmill speed was the same as that used for the maximal test and % incline was adjusted to elicit the appropriate intensity according to the ACSM equation for $\dot{V}O_2$ during treadmill running (6). Intensity was verified using the heart rate reserve method.

Isolation of $CD34^+$ cells. PBMCs were isolated from 30 ml EDTA-anticoagulated blood samples using density gradient centrifugation. $CD34^+$ enriched PBMCs were obtained using immunomagnetic selection according to the manufacturer's instructions (Stemcell Technologies). Briefly, the final PBMC pool was resuspended in PBS + 2% FBS at a density of 2×10^8 cells/ml. The $CD34^+$ selection antibody cocktail was added (100 µl/ml) to the PBMC suspension and incubated at room temperature for 15 min. Magnetic nanoparticles were then added (100 µl/ml) and incubated for 10 min, and PBS + 2% FBS was added to bring suspensions to a final volume of 2.5 ml. Cells were then incubated in the selection magnet for 5 min. $CD34^+$ cells remain attached to the side of the tube, and $CD34^-$ cells were poured off in the supernatant. The magnetic incubation step was repeated, and $CD34^+$ and $CD34^-$ cell fractions were counted by hemocytometer. Flow cytometry analysis of the immunomagnetically-purified CD34+ and CD34- cell fractions indicated 59% purity in the positively-selected fraction. Importantly, we found no detectable $CD34^+$ cells in the CD34-depleted fraction. These values are comparable to or better than previously-published purity values for immunomagnetically-selected CD34+ cells from unmobilized adult peripheral blood. For example, the purity of CD34+ cells reported by Asahara et al. (13) was only 16%, and Schatteman et al. have reported

CD34+ cell purity values ranging from 20-50% (79, 183, 186). Finally, the percent yield of immunomagnetically purified CD34+ was not affected by acute exercise (~1% of total PBMCs both before and after exercise). In addition, PCR confirmed high expression of CD34 mRNA in $CD34^+$ enriched cells, with only faint expression detected in $CD34^-$ cells.

Detection of intracellular free radicals and inhibition of NADPH oxidase activity. NO measurements were performed in duplicate as we have described previously (109), with modifications to examine freshly-isolated cells in a 96-well format. Briefly, 1.5×10^5 cells stained with 10 μM DAF-FM diacetate for determination of NO levels and 10 μM dihydroethidium (DHE) for determination of $O_2^{\cdot-}$ levels (Molecular Probes). Cells were also incubated with or without 1 mM apocynin (a pharmacologic NADPH oxidase inhibitor) to determine the mechanistic role of NADPH oxidase underlying the effects of acute exercise or differences between trained and sedentary groups. Cells were incubated with DAF-FM, DHE and drug or vehicle treatments in a final volume of 150 μl serum-free PBS for 60 min at 37°C. Excess DAF-FM and DHE were removed by centrifugation at 500*g* for 5 min and cells were resuspended in 150 μl PBS. NO fluorescence was quantified using a fluorescent plate reader (Wallac Victor^2 1400, Perkin Elmer) using excitation and emission filters of 488 and 535 nm, respectively. $O_2^{\cdot-}$ fluorescence was measured using excitation and emission filters of 543 and 620 nm, respectively. NO and $O_2^{\cdot-}$ fluorescence values were normalized to cell number and are expressed relative to the mean for the endurance-trained group's baseline $CD34^+$ cells. Intra-assay coefficients of variation for NO and $O_2^{\cdot-}$ were 3.6% and 2.6%, respectively. Because each assay

was performed on different days for each subject, inter-assay coefficients of variation were determined on blank samples (PBS only) to document the day-to-day variability in arbitrary/background fluorescence. The inter-assay coefficients of variation were 5.0% and 2.7% for NO and $O_2^{\cdot -}$ assays, respectively, indicating good day-to-day reliability of the assays. For validation of the fluorescent probes, unfractionated PBMCs and in $CD34^+$-enriched cells were treated with a NOS inhibitor (L-NAME, 300 uM) and a SOD mimetic (Tempol), which reduced the DAF and DHE fluorescence signals, respectively, to ~10-20% of basal levels (n = 3 in pilot experiments). Conversely, for positive controls, treatment of cells with NO and $O_2^{\cdot -}$ donor 3-morpholino-sydnomine dose-dependently increased the DAF and DHE signals in by ~1200-1800 fold compared to basal levels.

Semiquantitative reverse-transcriptase PCR. mRNA levels of endothelial nitric oxide synthase (eNOS), NADPH oxidase subunits $gp91^{phox}$ and $p47^{phox}$, superoxide dismutases (SOD; SOD1 and SOD2) and glutathione peroxidase-1 (GPX-1) were measured exactly as described previously (109). Additionally, cells were examined for expression of CD34 (forward primer: TGAAAAAGCTGGGGATCCTAGA, reverse primer: TCCCAGGTCCTGAGCTATAGCC), vascular endothelial growth factor (VEGF) (forward primer: AAGGAGGAGGGCAGAATCAT, reverse primer: ATCTGCATGGTGATGTTGGA), and inducible NOS (iNOS; forward primer: GGCCGCAGAGAACTCAGCCTCA, reverse primer: CTCAAAACAGCCGCTTCCCCAGAA). PCR products were electrophoresed on 1.5% agarose gels and visualized under UV light. Band intensities were quantified using imageJ and normalized to the signal for 18S as a reference gene. Data are

expressed relative to the endurance-trained group's normalized baseline value (set at 100%).

Statistics. Data were analyzed using a two-factor [group (trained or sedentary) × time (baseline and after exercise)] repeated measures ANOVA. Assumptions of normality and homoscedasticity were verified for all data. The criterion for statistical significance was $P \leq 0.05$. Data are presented as means ± SEM.

RESULTS

Subject characteristics. Endurance-trained and sedentary groups were successfully matched for age and BMI, but differed substantially (~33%; $P < 0.05$) in terms of $\dot{V}O_2max$ (Table 4.1). For one subject in the endurance-trained group, only baseline data were available (thus n = 9 for endurance-trained subjects after exercise).

Intracellular NO and $O_2^{\cdot -}$

$CD34^+$ cells. Sedentary subjects had 26% higher baseline NO levels compared to endurance-trained subjects ($P < 0.05$, Fig 4.1A) and NO levels in the trained group tended to increase with acute exercise ($P = 0.055$; Fig 4.1A). Apocynin treatments had no effect on NO levels of $CD34^+$ cells in either group ($P > 0.05$). Analysis of main effects revealed greater NO in $CD34^+$ cells of the sedentary group with data collapsed across treatment conditions ($P = 0.005$). The sedentary group also had higher $O_2^{\cdot -}$ levels at baseline ($P < 0.05$; Fig 4.1B). $O_2^{\cdot -}$ levels increased in the endurance-trained group with acute exercise ($P < 0.05$) but did not change in the sedentary group ($P > 0.05$). Treatment with apocynin reduced $O_2^{\cdot -}$ levels in the sedentary group both before and after exercise relative to their baseline vehicle-control condition ($P < 0.05$) such that baseline group differences were completely

abolished ($P < 0.05$). However, apocynin treatment had no effect on $O_2^{\cdot-}$ in $CD34^+$ cells from the trained group.

$CD34^-$ cells. The sedentary and endurance-trained groups had similar NO levels at baseline (Fig 4.2A). In the trained group, there was no effect of acute exercise on NO levels, although apocynin (~45%, $P < 0.05$) increased NO levels both at baseline and after acute exercise. Acute exercise tended to increase NO in the sedentary group by 38% ($P = 0.10$), and this increase resulted in a significant difference between the groups after acute exercise (56% greater NO levels in the sedentary group; $P < 0.05$). Apocynin increased NO levels in $CD34^-$ cells from the sedentary group by 46% at baseline and by 41% after acute exercise compared to their baseline vehicle-control condition (both $P < 0.05$). At baseline, NO levels increased with apocynin treatment to a greater extent in $CD34^-$ cells of the sedentary group compared to the trained group (group difference of 40%, $P < 0.05$). The difference between the groups was not statistically significant in apocynin-treated $CD34^-$ cells after acute exercise. There were no significant effects of training status, acute exercise, or apocynin on $O_2^{\cdot-}$ levels in $CD34^-$ cells (Fig 4.2B).

Gene expression

$CD34^+$ cells. Endurance-trained subjects had ~2-fold greater eNOS mRNA levels in $CD34^+$ cells compared to sedentary subjects at baseline ($P < 0.05$), and a similar trend was observed after acute exercise ($P = 0.08$; Fig 4.3A). iNOS mRNA levels in the sedentary group were ~2-fold greater than those of the trained group at baseline and after acute exercise (both $P < 0.05$; Fig 4.3B). VEGF mRNA levels were (~20%) higher in the trained group at baseline ($P < 0.05$) but the difference between the two

groups was not significant after acute exercise (Fig 4.3C). SOD1 mRNA levels were ~25% and 40% higher in cells of the trained group before and after acute exercise, respectively ($P < 0.05$), with the sedentary group having a significant acute exercise-induced decrease in SOD1 mRNA (Fig 4.3D). There were no significant effects of acute exercise or training status on SOD2 or GPX1 mRNA levels (Fig 4.3E and F). $p47^{phox}$ mRNA levels were higher in the trained subjects compared to the sedentary group at baseline (~40%, $P < 0.05$) and after acute exercise (~50%, $P < 0.05$; Fig 4.3G). Both groups had significant acute exercise-induced decreases in $p47^{phox}$ mRNA levels ($P < 0.05$). $gp91^{phox}$ mRNA levels in $CD34^+$ cells were similar between groups but differed after exercise by ~40% ($P < 0.05$; Fig 4.3H). Both groups had significant exercise-induced reductions in $gp91^{phox}$ mRNA levels ($P < 0.05$).

$CD34^-$ cells. eNOS mRNA levels were ~25% lower in the sedentary group at baseline and increased with acute exercise in the sedentary group ($P < 0.05$, Fig 4.4A). However, there were no differences in iNOS mRNA levels between groups or effects of acute exercise for either group (Fig 4.4B). Endurance-trained subjects had ~25% higher baseline VEGF mRNA levels than the sedentary group at baseline ($P < 0.05$, Fig 4.4C), and groups had similar VEGF mRNA levels after acute exercise. There were no differences between groups in SOD1, SOD2, or GPX1 (Fig 4.4D-F) at baseline, and acute exercise-induced increases were only evident in the sedentary group ($P < 0.05$ for SOD1 and SOD2, $P = 0.08$ for GPX1). There were no differences between groups or with acute exercise in expression of $p47^{phox}$ or $gp91^{phox}$ (Fig 4.4G-H).

DISCUSSION

The major findings in the CD34$^+$ cell fraction are (i) intracellular NO and $O_2^{\bullet-}$ levels are higher in sedentary than endurance-trained men; (ii) the effect of training status on $O_2^{\bullet-}$, but not NO levels, is NADPH oxidase-dependent; and (iii) mRNA levels of a number of angiogenic and antioxidant genes are lower in sedentary than trained men. Together, these findings point to increased nitro-oxidative stress in the sedentary state. Importantly, these data were obtained from a carefully selected and screened study sample of healthy, lean, young men with low risk for CV disease. Thus, the molecular changes in CD34$^+$ cells from sedentary subjects apparently occur associated with impaired angiogenic potential in circulating CD34$^+$ cells may occur very early in the disease-related process associated with a sedentary lifestyle.

Our finding of higher intracellular NO in CD34$^+$ cells of the sedentary group was contrary to our original hypothesis. Data from human and animal studies indicate that maintaining optimal levels of intracellular NO in vascular endothelial cells is a key mechanism by which exercise improves CV health (78, 126). Additionally, we and others have previously shown that cultured EPCs seem to exhibit similar NO-related biology compared to fully differentiated endothelial cells. For example, cultured angiogenic cells upregulate endothelial and angiogenic genes in response to shear stress (254). EPCs of CAD patients had undetectable eNOS and impaired migratory capacity that was reversed upon treatment with a NO donor (88). We previously reported that cultured EPC colonies from endurance-trained individuals had higher NO levels compared to sedentary individuals (109). Together, these previous data provided a strong rationale for our original hypothesis that trained men would have higher NO in circulating CD34$^+$ cells.

These previous investigations were based on the premise that eNOS is the primary source of NO in endothelial cells, and, by extension, in angiogenic cells that take on an endothelial phenotype in culture. However, the iNOS isoform also produces NO in large quantities in hematopoietic cells (213). The expression and activity of iNOS relative to eNOS can determine whether NO performs beneficial *physiological* functions (e.g. vasodilation in ECs, progenitor/angiogenic cell migration and homing) or harmful *pathophysiological* functions (e.g. inflammation, nitro-oxidative damage to cell components, and apoptosis) (157, 191, 222). These adverse consequences of high NO are amplified in the presence of excess $O_2^{\bullet -}$, as the interaction of these two radicals results in the rapid and spontaneous formation of peroxynitrite (213). Peroxynitrite increases iNOS expression (32) and promotes uncoupling of the eNOS reaction (61). Thus, the present findings of greater iNOS gene expression, lower eNOS gene expression, greater NO levels, and greater NADPH oxidase-derived $O_2^{\bullet -}$ production in the sedentary group are suggestive of a state of increased nitro-oxidative stress in $CD34^+$ cells of sedentary individuals. However, our cross-sectional study design allows us to conclude that we have identified an association, but not necessarily a cause-effect relationship, between a sedentary lifestyle and increased nitro-oxidative stress.

It is also possible that our present finding of higher levels of NO in cells from the sedentary group was in contrast with our previous finding of higher NO levels in cultured EPCs from trained compared to sedentary men (109) because of the difference between cultured and freshly-isolated cell characteristics. Our use of freshly-isolated cells in the present study is a very different approach compared to

using cultured PBMC-derived EPCs grown in a powerful endothelial growth environment (i.e. exposure of high concentrations to known endothelial growth factors for 5 days), as in our previous study. It is reasonable to suggest that cells from exercise-trained subjects may be primed for endothelial-directed differentiation under angiogenic growth conditions, owing to their higher expression of eNOS (in ref. 20, and replicated in both cell fractions of the present study) and VEGF gene expression.

It was surprising that NO and $O_2^{\cdot-}$ levels increased with exercise in $CD34^+$ cells from the trained group but not the sedentary group, which also contrasted with our previous data (109). An important aspect of our study with respect to the acute exercise data is that we obtained samples at only one time point after exercise. A time course experiment is warranted to determine whether NO and/or $O_2^{\cdot-}$ in freshly-isolated $CD34^+$ cells are unaffected by acute exercise in sedentary individuals, or whether training status determines the temporal nature of the response.

Treatment of $CD34^+$ cells with apocynin reduced intracellular $O_2^{\cdot-}$ in the sedentary group and a normalization of the baseline $O_2^{\cdot-}$ difference between groups, suggesting that the training-related difference in $O_2^{\cdot-}$ observed at baseline was a result of increased NADPH oxidase enzymatic activity in the sedentary group. Excessive NADPH oxidase-derived $O_2^{\cdot-}$ has been implicated in oxidative stress-related dysfunction of progenitor cell angiogenic activities (61). Our data provide the first evidence that NADPH oxidase activity is elevated in freshly-isolated $CD34^+$ cells of sedentary individuals, and it is important to emphasize that because we matched groups for age and BMI, we are confident that we have isolated the effect of

chronic exercise training to the extent possible given the cross-sectional study design. Nevertheless, these findings will require confirmation in a future prospective study.

The major findings in the CD34$^-$ cell fraction are (i) training status modifies the regulatory effect of NADPH oxidase enzyme activity on intracellular NO concentrations, as indicated by the effect of apocynin on NO being different between the sedentary and trained groups; (ii) regular endurance exercise is associated with enhanced angiogenic gene expression (i.e., VEGF and eNOS); and (iii) acute exercise increases antioxidant gene expression in CD34$^-$ cells of sedentary individuals.

The greater response of NO levels to apocynin treatment in the sedentary group than in the trained group is possibly indicative of greater basal NADPH oxidase enzymatic activity in the sedentary group. This finding is in line with our previous observations in putative EPC colonies, most of which are CD34$^-$ (109). Thus, further research is required to link the role of NADPH oxidase enzymatic activity and its regulatory effects on NO levels with angiogenic actions of particular CD34$^-$ cellular subsets. In addition, our data suggest that the angiogenic properties of CD34$^-$ cells may be enhanced by acute and chronic endurance exercise, as indicated by the higher levels of basal VEGF and eNOS mRNA in trained compared to sedentary subjects, and also by the acute exercise-induced increased in antioxidant genes in CD34$^-$ cells of sedentary subjects. However, the CD34$^-$ cells as a whole should probably not be thought of as angiogenic per se, as these cells are mostly white blood cells (T-cells, monocytes, etc) with primarily immune function, and it would be interesting to know which cell populations within the CD34$^-$ fraction are driving the present results. A reasonable hypothesis for future research is that the functions of angiogenic CD34$^-$

monocyte and T-cell fractions (15, 34, 79, 120, 122) may benefit from acute (based on our finding of increased antioxidant gene expression) and chronic (based on our finding of higher eNOS and VEGF gene expression) endurance exercise.

Limitations. It would have been advantageous to have selected additional subpopulations within the $CD34^+$ fraction, e.g. $CD34^+$/$VEGFR2^+$ or $CD34^+$/$CD45^-$ EPCs, however cell yields in pilot studies were too low to perform NO or $O_2^{\cdot -}$ assays. Thus, we chose to examine only two subfractions based on the presence or absence of the CD34 antigen, which yielded an appropriate number of cells to work with and seemed reasonable given their accepted role as proangiogenic cells under certain conditions. We did not measure all aspects of angiogenic function (e.g., *in vitro* capillary formation, migration, and assessments of target genes at the protein level), and clearly data generated from such assays would have aided the interpretation of the unexpected finding of higher NO levels in $CD34^+$ cells of the sedentary group.

Conclusions. The present study provides the first evidence that physical inactivity is associated with increased nitro-oxidative stress in $CD34^+$ cells, and it appears that the notion that higher levels of NO are necessarily associated with beneficial cellular outcomes will require further investigation. In particular, the relative contribution of eNOS vs. iNOS activities to $CD34^+$ cell-mediated vascular endothelial maintenance needs to be examined. As we have investigated these outcomes in healthy young men at low risk for CV disease, we have isolated the effects of exercise training independent of other confounding factors. Thus, we anticipate that the information provided by our study will be particularly useful for the development of therapeutic applications of $CD34^+$ cells in regenerative medicine.

The efficacy of cell therapy trials may be improved if exercise training as a strategy to reduce nitro-oxidative stress were employed in conjunction with cell infusions. However, further investigation is required to confirm the nitro-oxidative stress at the functional level (e.g. nitration of proteins) in $CD34^+$ cells. Overall, our data provide strong a rationale for further research to clarify the mechanisms of exercise-induced improvements in the vascular repair capacity of circulating $CD34^+$ PBMCs.

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Table 4.1. Subject characteristics.

	Endurance-Trained (n = 10)	Sedentary (n = 10)
Age (y)	25 ± 1	25 ± 1
BMI ($kg \cdot m^{-2}$)	22.1 ± 1	23.9 ± 1
Body fat (%)	7.1 ± 0.4	14.9 ± 1.3*
Glucose ($mg \cdot dl^{-1}$)	78 ± 2	79 ± 4
Cholesterol ($mg \cdot dl^{-1}$)	165 ± 6	171 ± 12
HDL-C ($mg \cdot dl^{-1}$)	65 ± 4	54 ± 4*
LDL-C ($mg \cdot dl^{-1}$)	88 ± 4	99 ± 13
TC/HDL	2.6 ± 0.1	3.4 ± 0.4
LDL/HDL	1.4 ± 0.1	2.0 ± 0.4*
VLDL-C ($mg \cdot dl^{-1}$)	11 ± 0.9	19 ± 2*
Triglycerides ($mg \cdot dl^{-1}$)	57 ± 5	95 ± 9*
SBP (mm Hg)	121 ± 3	119 ± 2
DBP (mm Hg)	74 ± 2	76 ± 4
MAP (mm Hg)	90 ± 2	91 ± 2
$\dot{V}O_2$max		
$l \cdot min^{-1}$	4.82 ± 0.13	3.68 ± 0.14*
$ml \cdot kg^{-1} \cdot min^{-1}$	70.3 ± 0.9	46.9 ± 1.5*
$ml \cdot kg\ FFM^{-1} \cdot min^{-1}$	75.6 ± 1.1	55.1 ± 1.5*

*Statistically significant difference between groups ($P < 0.05$). Abbreviations: BMI, body mass index; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein-cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; $\dot{V}O_2$max, maximal oxygen uptake.

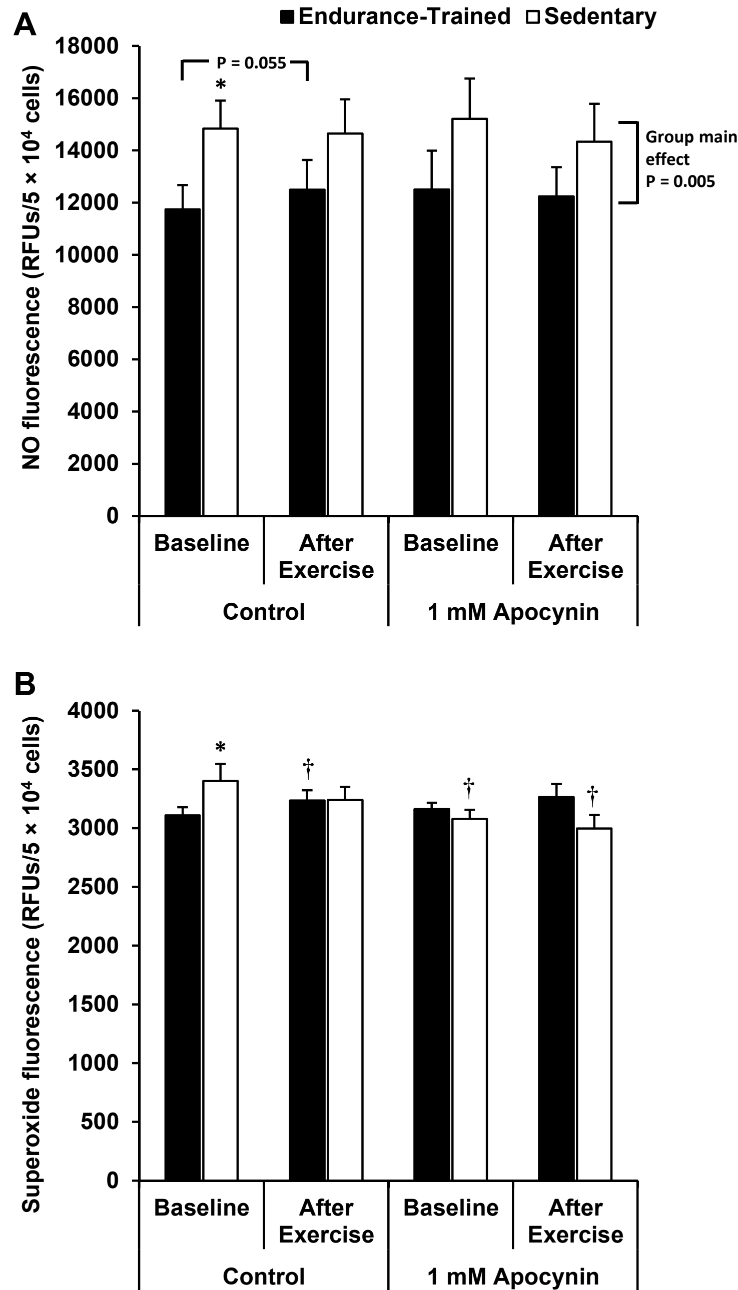

Figure 4.1. Nitric oxide (NO; panel A) and superoxide (panel B) fluorescence in $CD34^+$ cells of endurance-trained and sedentary men before and after acute exercise. *Statistically significant difference between groups within the given experimental condition ($P < 0.05$); †Statistically significant within-group change relative to baseline control sample ($P < 0.05$).

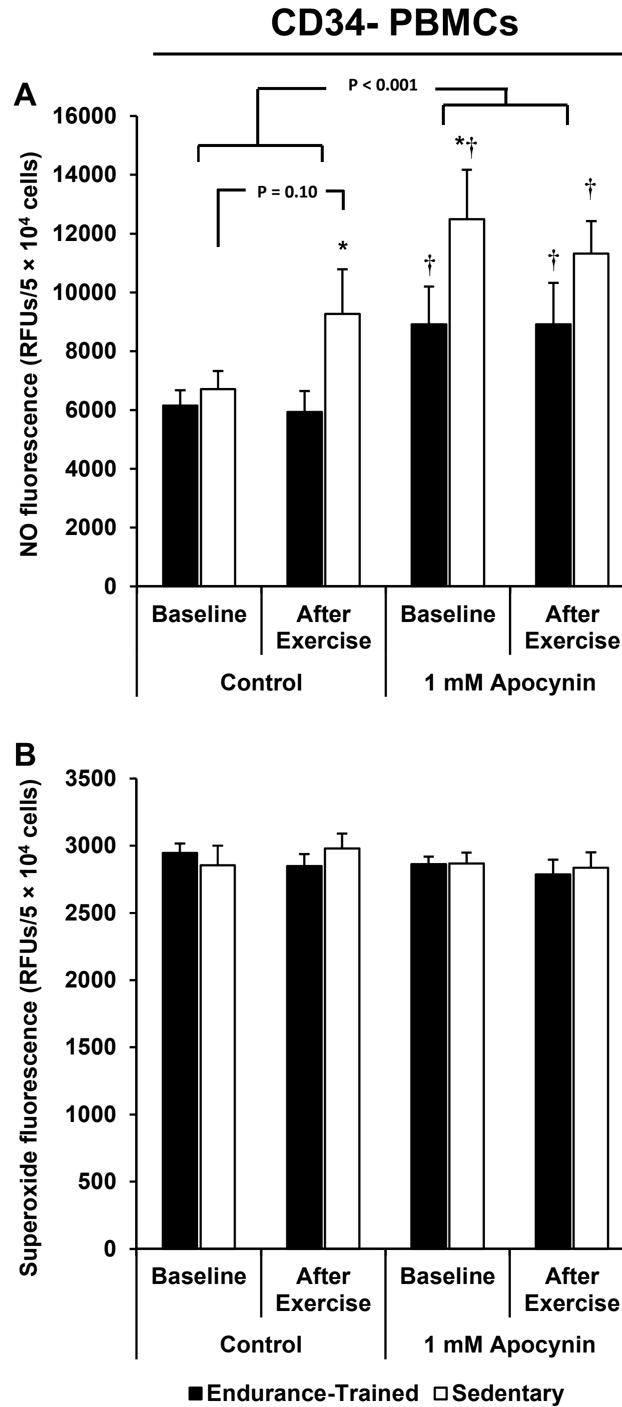

Figure 4.1. Nitric oxide (NO; panel A) and superoxide (panel B) fluorescence in $CD34^-$ cells of endurance-trained and sedentary men before and after acute exercise. *Statistically significant difference between groups ($P < 0.05$) within the given experimental condition; †Statistically significant within-group change relative to baseline control sample ($P < 0.05$).

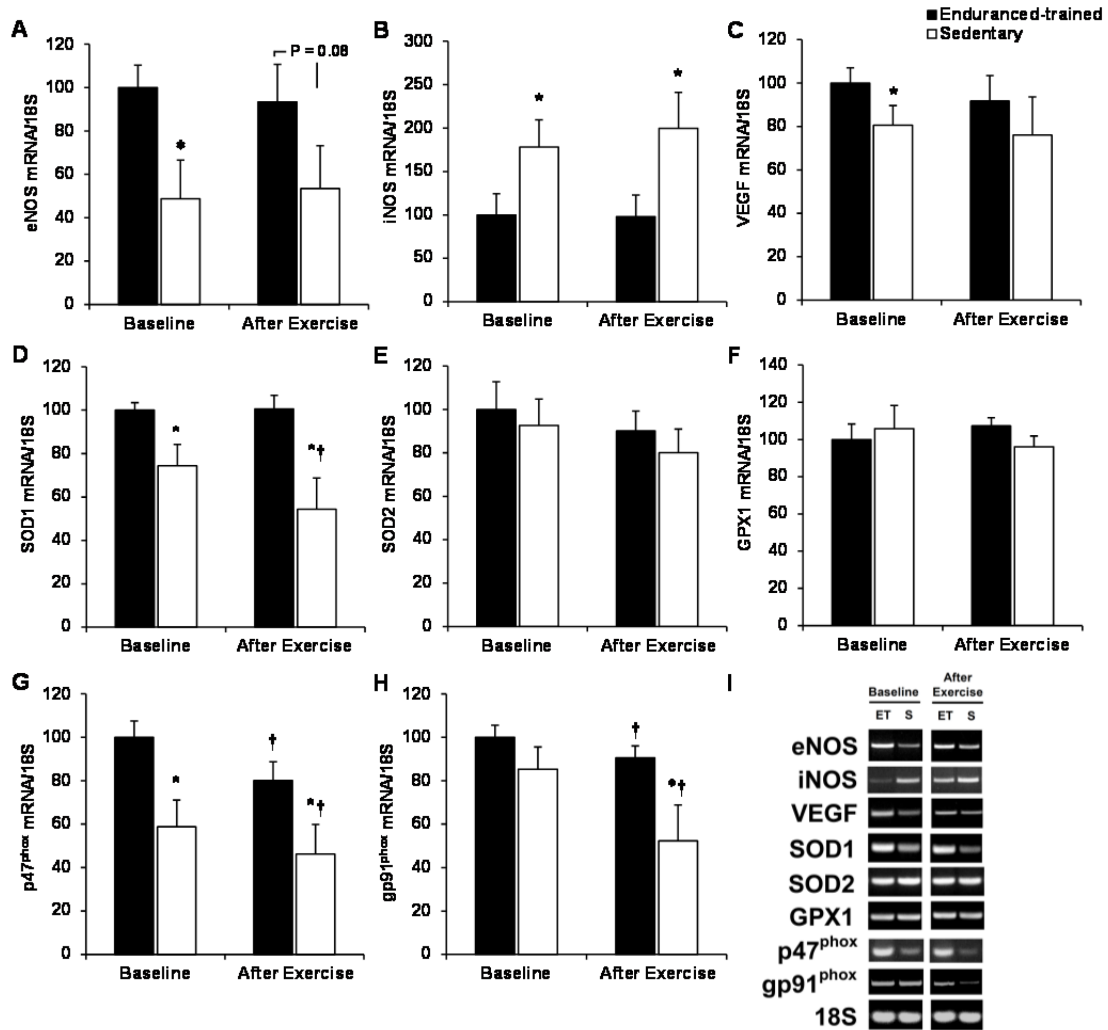

Figure 4.3. mRNA levels of eNOS (A), iNOS (B), VEGF (C), SOD1 (D), SOD2 (E), GPX-1 (F), p47phox (G), and gp91phox (H) in $CD34^{+}$ cells of endurance-trained and sedentary men before and after acute exercise. (I) RT-PCR agarose gel electrophoresis products shown are representative of data presented in panels A-H; separate images from the same gel are shown for each target gene. Abbreviations: ET = endurance-trained group; S = sedentary group. *Statistically significant difference between groups within the given experimental condition ($P < 0.05$); †Statistically significant within-group change relative to baseline control sample ($P < 0.05$).

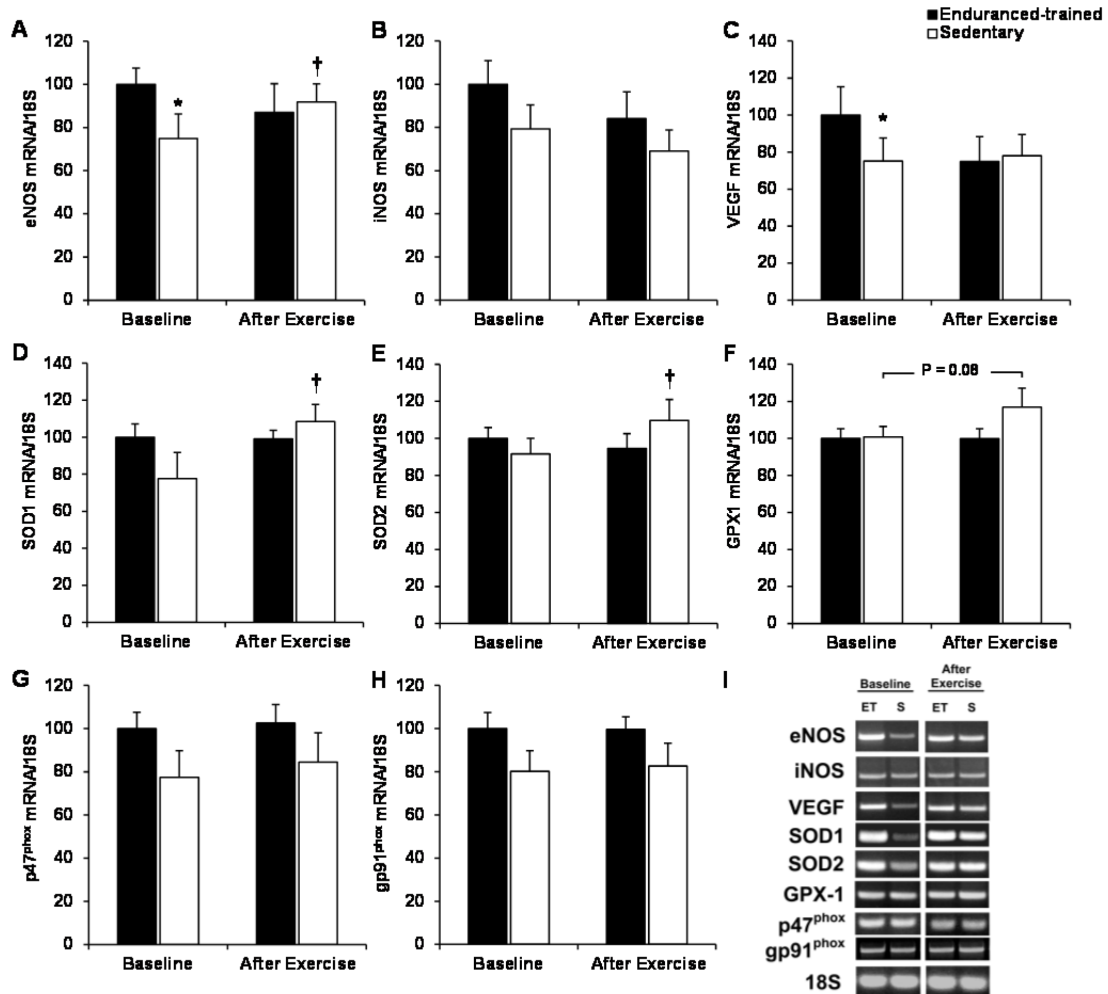

Figure 4.4. mRNA levels of eNOS (A), iNOS (B), VEGF (C), SOD1 (D), SOD2 (E), GPX-1 (F), p47phox (G), and gp91phox (H) in $CD34^-$ cells of endurance-trained and sedentary men before and after acute exercise. (I) RT-PCR agarose gel electrophoresis products shown are representative of data presented in panels A-H; separate images from the same gel are shown for each target gene. Abbreviations: ET = endurance-trained group; S = sedentary group. *Statistically significant difference between groups within the given experimental condition ($P < 0.05$); †Statistically significant within-group change relative to baseline control sample ($P < 0.05$).

Chapter 5: Prior Endurance Exercise Prevents Postprandial Lipemia-Induced Increases in Reactive Oxygen Species in Circulating CD31^{+} Cells

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Prior Endurance Exercise Prevents Postprandial Lipemia-Induced Increases in Reactive Oxygen Species in Circulating CD31^{+} Cells

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ABSTRACT

OBJECTIVE: We hypothesized that prior exercise would prevent postprandial lipemia (PPL)-induced increases in intracellular reactive oxygen species (ROS) in three distinct circulating angiogenic cell (CAC) subpopulations.

METHODS and RESULTS: $CD34^+$, $CD31^+/CD14^-/CD34^-$, and $CD31^+/CD14^+/CD34^-$ CACs were isolated from blood samples obtained from ten healthy men before and 4 hr after ingesting a high fat meal with or without ~50 min of prior endurance exercise. Significant PPL-induced increases in ROS production in both sets of $CD31^+$ cells were abolished by prior exercise. Experimental *ex vivo* inhibition of NADPH oxidase activity and mitochondrial ROS production indicated that mitochondria were the primary source of PPL-induced oxidative stress. The attenuated increases in ROS with prior exercise were associated with increased antioxidant gene expression in $CD31^+/CD14^-/CD34^-$ cells and reduced intracellular lipid uptake in $CD31^+/CD14^+/CD34^-$ cells. These findings were associated with systemic cardiovascular benefits of exercise, as serum TG, OxLDL, and plasma EMP concentrations were lower in the exercise trial than the sedentary trial.

CONCLUSIONS: Prior exercise completely prevents PPL-induced increases in ROS in $CD31^+/CD14^-/CD34^-$ and $CD31^+/CD14^+/CD34^-$ cells. The mechanisms underlying the effects of exercise on CAC function appear to vary among specific CAC types.

INTRODUCTION

Ingestion of a high-fat meal increases serum triglyceride concentrations (TG), induces systemic oxidative stress, and impairs vascular endothelial function (220, 237). As the majority of individuals in Western society are in a postprandial state most of the time (196), routine exposure to postprandial lipemia (PPL) and the associated endothelial dysfunction may affect the development of cardiovascular (CV) disease (7). PPL-induced vascular endothelial dysfunction is thought to be mediated by oxidative stress resulting from an increased lipid load within the cell, in turn leading to increased oxidative metabolism and excess production of reactive oxygen species (ROS) (16, 239). The primary consequences of increased ROS production are nitric oxide (NO) scavenging and uncoupling of the endothelial NO synthase (eNOS) enzyme (159), ultimately resulting in reduced NO bioavailability, impaired NO-dependent vasodilatory function, and, with chronic exposure, a pro-atherogenic cellular environment within the vessel wall (239).

Prior exercise effectively reduces PPL (164, 239) and the accompanying vascular endothelial dysfunction (160, 221). The mechanisms underlying the protective effects of exercise involve up-regulation of systemic (221) and intracellular (16, 23, 239) antioxidant defenses that enable the preservation of NO bioavailability and vascular function in the face of a high-fat challenge. However, it is unknown whether these mechanisms are uniform throughout all CV cells and tissues.

Circulating angiogenic cells (CACs) are now understood to be important for the maintenance of a healthy endothelium (250). CAC number is associated with CV disease incidence and risk factors, such as obesity, physical activity, and diabetes (52,

56, 137, 212, 251). However, there are few published data on the functional responses of CACs to lifestyle factors. The CAC populations that have received the most attention are bone marrow-derived $CD34^+$ progenitor cells that co-express endothelial markers (12, 13). However, although cells expressing the endothelial antigen CD31 (34, 114, 115) and/or the monocyte antigen CD14 (15, 59, 79, 141, 178, 184) represent important CAC subpopulations, the effects of exercise and other lifestyle factors on functional aspects of these cell populations have not been studied. Finally, several studies have shown that PPL causes inflammation and ROS production in leukocytes (5, 71, 101, 235), but whether these effects are uniform among leukocyte subsets (including CACs) or could be ameliorated by exercise is not known.

In the present study, we tested the effect of a high fat meal with or without a bout of prior endurance exercise on ROS and NO production in $CD34^+$, $CD31^+/CD14^-/CD34^-$, and $CD31^+/CD14^+/CD34^-$ CACs. We also attempted to gain mechanistic insight into the molecular source of PPL-induced ROS using pharmacological inhibition of NADPH oxidase- and mitochondria oxidative activity. We hypothesized that PPL would reduce intracellular NO levels and increase the production of ROS by NADPH oxidase and/or mitochondria, and that these effects would be attenuated by a single bout of exercise performed on the prior day. In addition, we assessed concentrations of serum TG, serum oxidized LDL-cholesterol (OxLDL), and plasma endothelial microparticles (EMPs) to determine if exercise reduces systemic PPL-induced oxidative stress and endothelial damage in conjunction with any effects on CACs.

METHODS

Overview of Experimental Procedures

Following a screening visit to obtain informed consent and assess maximal oxygen uptake ($\dot{V}O_2max$) and body composition [inclusion criteria: male, age 18-35 yr, normotensive, normolipidemic, normoglycemic, not taking any medications, and physically active (at least 4 hr/wk participation in endurance exercise)], subjects were subjected to PPL tests under two separate experimental conditions: control, in which no prior exercise was performed, and an exercise trial, in which subjects performed stationary cycling at 70% $\dot{V}O_2max$ until reaching an energy expenditure of 2.5 MJ (~50 min). Subjects remained sedentary for the entire 24 hr period preceding the PPL test. The exercise trial was completed ~15 hr prior to the PPL test. The order of treatments was randomized and balanced across the study subjects (n = 10), and treatments were scheduled 1 week apart. Subjects consumed a standardized meal the evening before the PPL test. The PPL test meal consisted of heavy whipping cream, chocolate syrup, sugar, and powdered milk, and provided ~1300 kcal (84% from fat). Additional details of the study protocol, including the exercise protocol and test meal macronutrient composition for each subject, are provided in the Supplemental Materials (Supplemental Tables 5.I and 5.II). The University of Maryland College Park Institutional Review Board approved all study procedures and subjects provided written informed consent.

Immunomagnetic Cell Separation

Peripheral blood mononuclear cell (PBMCs) were isolated by density gradient centrifugation (Ficoll; GE Healthcare), and separated into $CD34^+$, $CD31^+/CD14^-/CD34^-$, and $CD31^+/CD14^+/CD34^-$ cell fractions using an immunomagnetic cell

separation procedure (EasySep®, Stemcell Technologies) according to the manufacturer's instructions (additional details are provided in Supplemental Materials).

Experimental Inhibition of NADPH oxidase and Mitochondrial ROS Production

NADPH oxidase was inhibited using apocynin (Sigma), and mitochondria respiratory complex I was inhibited by rotenone (Sigma; additional details are provided in the Supplemental Materials).

Measurement of Intracellular NO and ROS, Intracellular Stored Neutral Lipid Assay, RNA Isolation and Assessment of Gene Expression, Serum Triglyceride and Oxidized LDL-cholesterol, Endothelial Microparticles

These protocols are described in the Supplemental Materials.

Statistics

Assumptions of homoscedasticity and normality were verified for all outcome measures. Data were analyzed using two-factor (condition × time) or three-factor (condition × time × drug) repeated measures ANOVA, where appropriate. Analyses of simple effects were used to determine differences between control and exercise treatments at specific time points. Total TG areas under the lipemia curves (TG AUC) were calculated using the trapezoid rule (140) and compared between control and exercise treatments using paired t-tests. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

Subject characteristics

Anthropometric and clinical characteristics of the study subjects are presented in Table 5.1.

Intracellular ROS and NO Levels

Reactive oxygen species. In $CD31^+/CD14^-/CD34^-$ cells, there was a ~30% increase in ROS with PPL in the control condition ($P < 0.05$), and this effect of PPL was completely absent in the prior exercise trial (Fig 5.1A). Apocynin increased ROS levels at both 0- and 4-hr time points in both trials ($P < 0.05$), but the magnitudes of these increases were significantly greater in the control trial compared to the trial with prior exercise ($P < 0.05$). The apocynin-induced increase in ROS levels in these and the two other cell types (presented below) was a surprising finding, given the hypothesis of the study that NADPH oxidase-inhibition would experimentally demonstrate that the PPL-induced increase in ROS would be driven by NADPH oxidase. When we observed that these findings were consistent and statistically significant after testing the first 5 subjects of the study, we attempted to determine if another cellular source of pro-oxidant activity could have compensated for NADPH oxidase inhibition by increasing ROS production. Thus, before testing the remaining participants, we examined the effects of allopurinol (a xanthine oxidase inhibitor), rotenone (a mitochondrial complex I inhibitor), and antimycin (a mitochondrial complex III inhibitor) in combination with apocynin (n = 3 pilot subjects). It was found that the most consistent inhibitor of the apocynin-induced increase in ROS was rotenone, i.e. ~60-100% of the increase could be eliminated by rotenone compared to ~20% by allopurinol and <10% by antimycin (data not shown). Therefore, rotenone was included in the experiments for the remaining subjects. The apocynin-induced

increases in ROS in $CD31^+/CD14^-/CD34^-$ cells were completely reversed by rotenone in both the control and prior exercise trials ($P < 0.05$). ROS levels were lower in the prior exercise trial compared to the control trial under all experimental conditions ($P < 0.05$).

In $CD31^+/CD14^+/CD34^-$ cells, PPL increased ROS by ~25% in the control trial ($P < 0.05$), and this increase was absent in the trial with prior exercise (Fig 5.1B). Apocynin substantially increased ROS levels at both time points during both trials ($P < 0.05$), and again this was completely reversed by rotenone treatment ($P < 0.05$). The magnitude of the increase in ROS levels with apocynin treatments and the degree of reduction by rotenone treatments were similar between control and exercise trials.

In $CD34^+$ cells, PPL induced a small (~10%), but statistically significant increase in ROS in the trial with prior exercise ($P < 0.05$), but no change was observed in the control trial (Fig 5.1C). NADPH oxidase inhibition with apocynin induced ~2-2.5 fold increases in ROS levels at 0 hr and 4 hr in both the control and prior exercise trials ($P < 0.05$), which was completely attenuated by co-incubation with rotenone ($P < 0.05$).

Nitric Oxide. There were no effects of PPL or apocynin on NO levels in either the prior exercise or control trial in $CD34^+/CD14^-/CD34^-$ cells (Fig 5.2A). In $CD34^+/CD14^+/CD34^-$ cells, PPL increased NO levels both with and without apocynin treatments by ~10-15% in the control trial ($P < 0.05$), but there were no effects of PPL or apocynin in the prior exercise trial (Fig 5.2B). PPL had no effect on NO in $CD34^+$ cells during the control trial, but induced a ~10% decrease in NO during the

trial with prior exercise ($P < 0.05$). This 10% reduction in NO was reversed upon treatment with apocynin (Fig 5.2C).

Intracellular Neutral Lipid Storage

Within the $CD34^+$ cells, PPL caused a significant increase in stored neutral lipids during the prior exercise trial ($P < 0.05$), but there was no change during the control trial (Fig 5.3). There were no significant PPL-induced changes in stored neutral lipids in $CD31^+/CD14^-/CD34^-$ cells in either trial. In $CD31^+/CD14^+/CD34^-$ cells, intracellular lipids increased significantly in the control trial ($P < 0.05$), but there was no effect of PPL in the prior exercise trial. There were no differences in basal (0 hr) stored neutral lipid levels between the control and prior exercise trials (data not shown).

Circulating TG, OxLDL, and EMP Concentrations

Fasting (i.e., 0-hr) serum concentrations of TG and OxLDL were not different between trials ($P > 0.05$; Fig 5.4). Serum TG concentrations increased significantly throughout the sampling period in both the control and prior exercise trials ($P < 0.05$), but the concentrations were lower ($P < 0.05$) at 1 and 3 hr, and tended to be lower at 4 hr ($P = 0.06$), during the prior exercise trial compared to the control trial (Fig 5.4A). TG AUC was significantly lower in the prior exercise trial compared to the control trial (Fig 5.4B, $P < 0.05$). There was a 28% increase in serum oxLDL concentration in the control trial ($P < 0.05$), and there was no change in oxLDL concentration in response to the high-fat meal with exercise performed on the prior day (Fig 5.4C and D). Plasma EMP markers of endothelial activation (Fig 5.5A) and endothelial apoptosis (Fig 5.5B) were lower in the prior exercise trial compared to the control

trial (55% and 30%, respectively; both $P < 0.05$), but were unaffected by the high-fat meal in either trial.

Gene Expression

Overall, the pattern of gene expression varied considerably among the three cell types in response to PPL with and without prior endurance exercise. The major finding was that prior exercise increased expression of the antioxidant genes superoxide dismutase (SOD)-1 and SOD2 in $CD31^+/CD14^-/CD34^-$ cells at both 0 hr and 4 hr time points, while SOD1 and SOD2 gene mRNA levels were not affected by prior exercise in either $CD31^+/CD14^-/CD34^-$ or $CD34^+$ cells. Additional data on mRNA levels of endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), vascular endothelial growth factor (VEGF), NADPH oxidase subunits $gp91^{phox}$ and $p47^{phox}$, and the oxidized LDL-cholesterol receptor-1 (LOX1) are presented in Supplemental Figures 5.II-IV.

DISCUSSION

The major finding of this study is that PPL increases intracellular ROS in $CD31^+/CD14^-/CD34^-$ and $CD31^+/CD14^+/CD34^-$ PBMCs, and this effect is prevented by performing endurance exercise on the prior day. Consistent with previous data indicating that postprandial oxidative stress is caused by an increase in mitochondria-derived ROS production (23, 239), our data implicate mitochondria as a possible source of PPL-induced increases in intracellular ROS in the $CD31^+$ CAC subfractions. In addition, we observed beneficial effects of exercise on PPL-induced changes in intracellular storage of neutral lipids and expression of genes involved in the functional status of CACs; however, there were adverse effects of PPL on $CD34^+$

cells (i.e., elevated ROS, reduced NO, and increased stored neutral lipid) in the prior exercise trial only. Therefore, our data suggest that the effects of exercise are not uniform among CAC subpopulations.

Exercise Protects Against High-fat Meal-induced Increases in Intracellular ROS of $CD31^{+}/CD14^{-}/CD34^{-}$ and $CD31^{+}/CD14^{+}/CD34^{-}$ Cells

We hypothesized that NADPH oxidase would play a role in the effects of PPL-induced oxidative stress, as PPL has been shown to increase OxLDL (220), and OxLDL increases NADPH oxidase expression in cultured CACs (135). However, in the present study apocynin treatment induced a dramatic increase in intracellular ROS levels in all three CAC types. We were able to determine that mitochondria up-regulate ROS production with acute apocynin treatments in all three cell types examined, as the apocynin-induced increase in ROS was completely reversed upon treatment with the complex I inhibitor rotenone. Further, and more importantly, the protective effects of exercise against PPL-induced ROS production in both $CD31^{+}$ cell fractions appeared to be mitochondria-dependent, as indicated by the return to 0-hr ROS levels in rotenone-treated 4-hr cells. However, we acknowledge that a limitation of our study is that we did not include a rotenone-only treatment condition. Thus, our interpretation that mitochondria might account for the PPL-induced ROS increase must be taken with caution, and will require confirmation in further experiments designed *a priori* to examine the role of mitochondria. It is also important to mention that in our preliminary experiments, apocynin at the chosen concentration (250 μM) decreased basal ROS production as measured by DCF fluorescence in unfractionated PBMCs (data not shown), and previous studies have

used similar or greater concentrations of apocynin to investigate the NADPH oxidase-driven components of cultured endothelial cell ROS production (150) and vascular endothelial dysfunction (46). Thus, the up-regulation of mitochondria-derived ROS by apocynin in our experiments appears to be a phenomenon specific to the CAC populations examined in the present study, and the biology of redox regulation does not appear to be uniform between vessel wall endothelial cells and CACs. Furthermore, it is interesting that the degree of the mitochondria-mediated increase in ROS was blunted with prior exercise in the $CD31^+/CD14^-/CD34^-$ fraction, indicating that in these cells there was an attenuation of mitochondrial ROS production in response to the high-fat meal, or there may have been an up-regulation of intracellular antioxidant defenses that resulted in enhanced scavenging of ROS. In line with the latter possibility, we also found increases in both SOD1 and SOD2 mRNA levels in $CD31^+/CD14^-/CD34^-$ cells with exercise (supplemental Fig 5.II). In $CD31^+/CD14^+/CD34^-$ cells, on the other hand, the PPL-induced increase in ROS was similarly blunted by prior exercise, and yet there were no effects of exercise on antioxidant gene expression (Supplemental Fig 5.III). However, in these cells prior exercise caused a significant reduction in the degree of neutral lipid storage with PPL, which would be expected to result in attenuated ROS production (239). Regardless, the critical finding of the present study is protection against PPL-induced oxidative stress in $CD31^+/CD14^-/CD34^-$ and $CD31^+/CD14^+/CD34^-$ angiogenic cells by prior exercise. These findings are especially important in light of recent reports that $CD31^+$ cells in bone marrow and peripheral blood are an important source of CACs for the maintenance of vascular endothelial integrity and are effective for cell-based

regenerative medicine therapy (114, 115). Our data suggest that exercise may enhance the ability of CD31^{+} CACs to cope with a physiological pro-oxidative challenge, and may therefore improve the efficacy of these cells for therapeutic treatment of CV diseases.

Exercise and PPL Have Minimal Effects on Intracellular NO in CACs

Surprisingly, there were few significant effects of PPL, exercise, or their interaction on intracellular NO levels in the three cell types examined in the present study. Given the critical role for NO in a number of CAC functions, we expected that NO would be reduced by PPL without prior exercise and that this reduction would be absent, or at least blunted, in the trial with prior exercise. Thus, the reduction in NO in CD34^{+} cells during the prior exercise trial was not anticipated. However, it was consistent with the gene expression data indicating that eNOS expression increased with PPL in the control but not the prior exercise trial (Supplemental Fig 5.IV). Similarly, there were no changes in NO levels in CD31^{+}/CD14^{-}/CD34^{-} cells, although in these cells mRNA levels of both NOS isoforms were significantly increased during the prior exercise trial compared to the sedentary trial (supplemental Fig 5.II). These effects at the mRNA level were not associated with changes at the functional level (i.e., intracellular NO). In CD31^{+}/CD14^{+}/CD34^{-} cells, conversely, PPL-induced increases in eNOS mRNA and in intracellular NO were observed both in the control trial, but not in the trial with prior exercise. However, it must be emphasized that the changes in NO in both CD34^{+} and CD31^{+}/CD14^{+}/CD14^{-} cells, while statistically significant, were relatively small (~10%), and therefore further research is needed to determine the physiological significance of these findings.

Role of Lipid Storage and Gene Expression: Evidence for CAC Subpopulation-specific Mechanisms Underlying Effects of Exercise

As mentioned above, we observed higher levels of in SOD1 and SOD2 mRNA levels in $CD31^+/CD14^-/CD34^-$ cells during the prior exercise trial, but differences between trials in these antioxidant genes in $CD31^+/CD14^+/CD34^-$. Conversely, there were no effects on lipid storage in $CD31^+/CD14^-/CD34^-$ cells, whereas in $CD31^+/CD14^+/CD34^-$ cells there was a significant reduction in the degree of PPL-induced lipid uptake with prior exercise. Furthermore, the expression profile of the angiogenic, pro-oxidative, and antioxidant genes we examined varied considerably in response to PPL and exercise among the three cell types we investigated (Supplemental Figures 5.II-IV). Therefore, it would appear that the mechanisms by which exercise and high-fat meal ingestion alter the angiogenic functions in CACs are not uniform among specific CAC subpopulations.

Exercise Blunts PPL-induced Increases in Serum TG and OxLDL and Reduces EMPs in Spite of PPL

Our finding of a reduction in TG AUC following ingestion of a high-fat meal with exercise performed on the prior day is consistent with numerous previous studies [for review, see Petitt and Cureton (164)]. An increase in serum OxLDL has been observed in response to ingestion of a high-fat meal (220), and to the best of our knowledge, this is the first study to report an exercise-induced protection against this effect. As OxLDL has been previously reported to cause dysfunction of cultured CACs through increased intracellular oxidative stress (135), the mechanisms underlying our findings of reduced intracellular ROS may have involved the reduced

exposure to circulating OxLDL after the high-fat meal with prior exercise. In addition, our data provide the first evidence that exercise can reduce circulating EMPs, a well-established marker of damaged vascular endothelium (94). Specifically, EMP populations indicating the presence of endothelial activation and endothelial apoptosis were lower in the exercise trial compared to the sedentary trial, even in the face of the high-fat challenge, which has previously been shown to increase circulating EMPs (60, 220). Thus, our TG, OxLDL, and EMP data are in line with (i) the known effects of a high-fat meal on TGs and on systemic oxidative stress, and (ii) the reduction in or prevention of PPL-induced vascular dysfunction (160, 221), which is mediated by oxidative stress (165, 166, 239).

CONCLUSIONS

In summary, we have shown that the PPL-induced increases in intracellular ROS production in $CD31^+$ cells are prevented by prior endurance exercise. Our experiments suggested mitochondria as the source of the PPL-induced increase in ROS, although future studies designed *a priori* to determine the role of mitochondria in PPL-induced ROS production in CACs are required for confirmation. These findings add to the strong and consistent evidence that the detrimental effects of PPL on the CV system are mediated by oxidative stress, and our direct measurements of the intracellular environment within human CACs indicate that these effects of PPL can be prevented by prior endurance exercise. Our data also provide novel insight into the understanding of how situations encountered in daily life, such as exercise and high-fat meal ingestion, might alter the endothelial repair capacity of CACs. These findings were linked to prior exercise-induced reductions in circulating TG, OxLDL,

and EMP concentrations during PPL, suggesting a systemic CV benefit of exercise even in the face of a high-fat challenge. Finally, our findings could provide important information for the ongoing efforts to optimize the application of CACs for therapeutic treatment of CV disease patients.

ACKNOWLEDGEMENTS

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Table 5.1. Subject Characteristics.

	Mean	SEM
Age (yr)	27	0.9
BMI (kg/m^2)	24.6	0.7
Body Fat (%)	15.1	1.2
Glucose (mg/dl)	82	2.2
Cholesterol (mg/dl)	163	5.8
HDL-C (mg/dl)	54	3.2
LDL-C (mg/dl)	93	3.7
VLDL-C (mg/dl)	16	1.7
TG (mg/dl)	78	8.1
SBP (mm Hg)	120	3.0
DBP (mm Hg)	75	1.8
MAP (mm Hg)	90	1.8
$\dot{V}O_2max$ (L/min)		
L/min	3.75	0.2
ml/kg/min	48.0	2.2

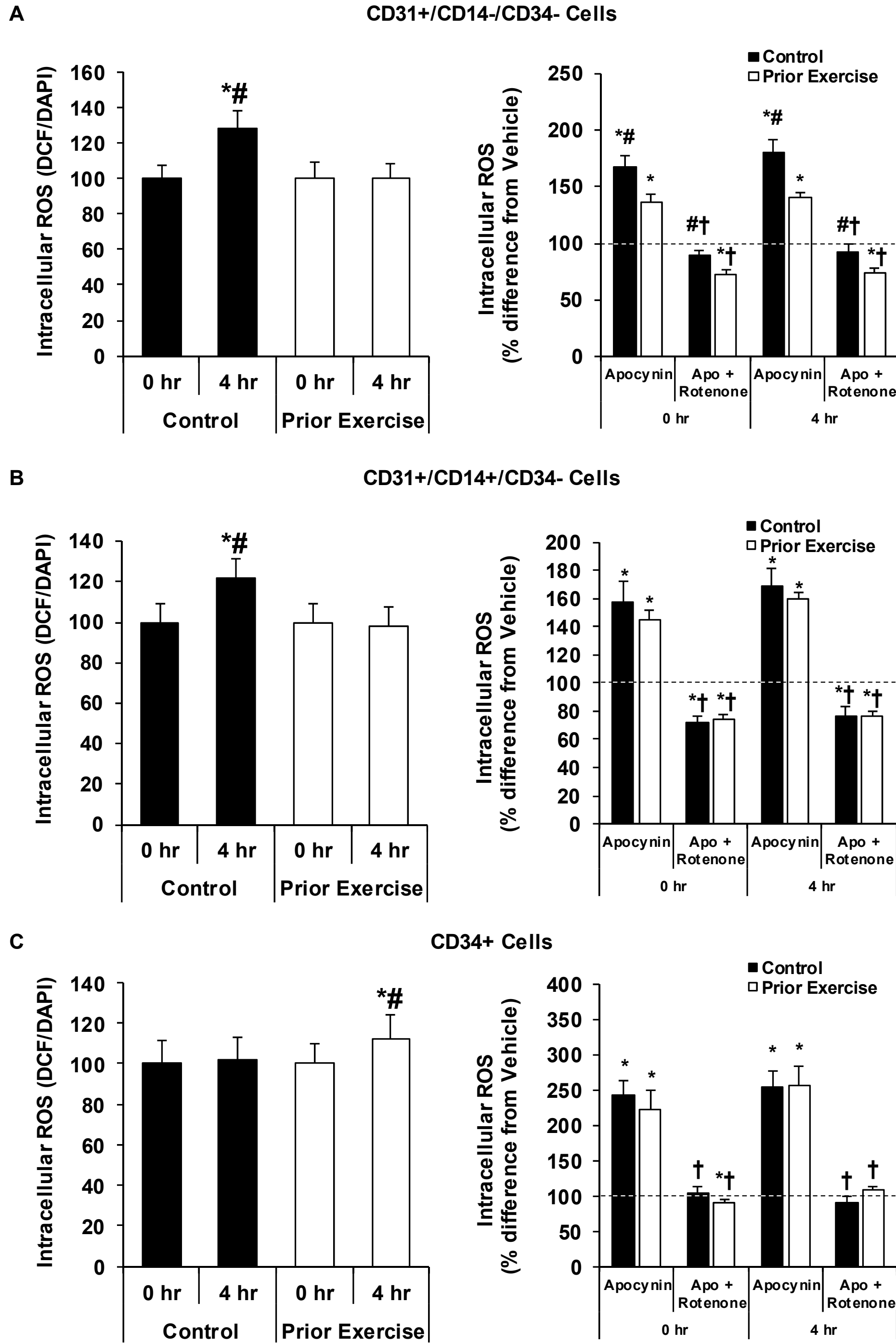


Figure 5.1. Effects of PPL with and without prior endurance exercise (left panels) and apocynin and rotenone treatments (right panels) on intracellular reactive oxygen species in $CD31^{+}/CD14^{-}/CD34^{-}$ (A), $CD31^{+}/CD14^{+}/CD34^{-}$ (B), and $CD34^{+}$ (C) CACs. Horizontal dashed line at 100% indicates control (vehicle) condition. *Statistically significant difference from 0-hr control (vehicle) condition ($P < 0.05$). #Magnitude of change from 0-hr control (untreated) condition is significantly different between control and prior exercise trials ($P < 0.05$). †Statistically significant difference between rotenone and apocynin-treated cells ($P < 0.05$).

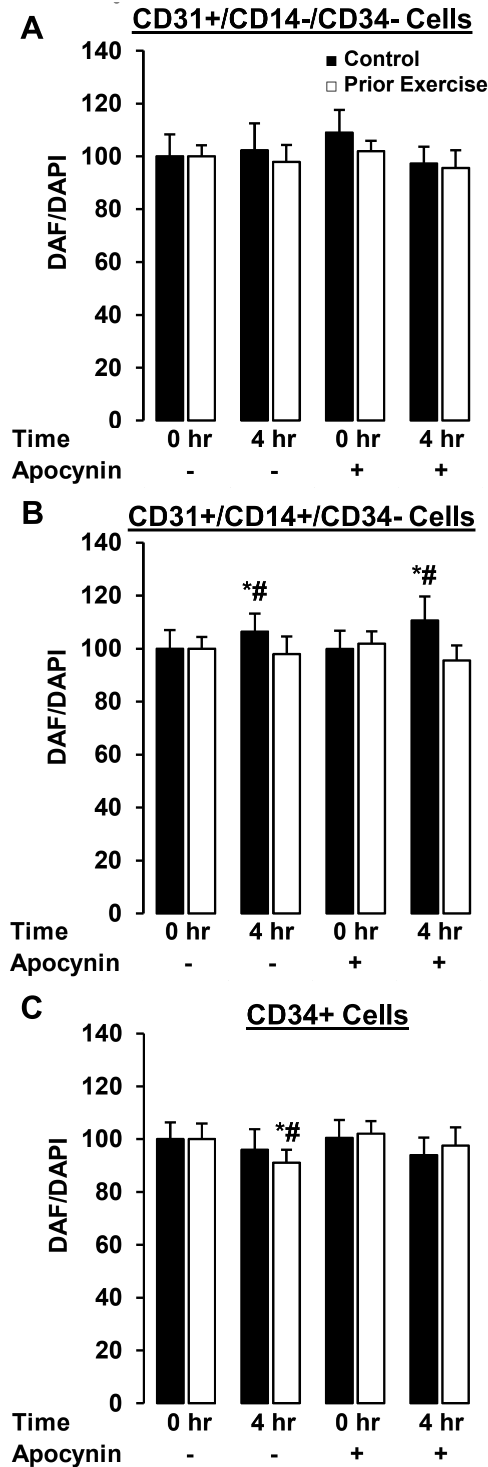


Figure 5.2. Effects of PPL and apocynin treatment with and without prior endurance exercise on intracellular nitric oxide in CD31+/CD14-/CD34- (A), CD31+/CD14+/CD34- (B), and CD34+ (C) circulating angiogenic cells. *Statistically significant difference from 0-hr control (vehicle) condition ($P < 0.05$). #Change from 0-hr control (untreated) condition is significantly different between trials ($P < 0.05$).

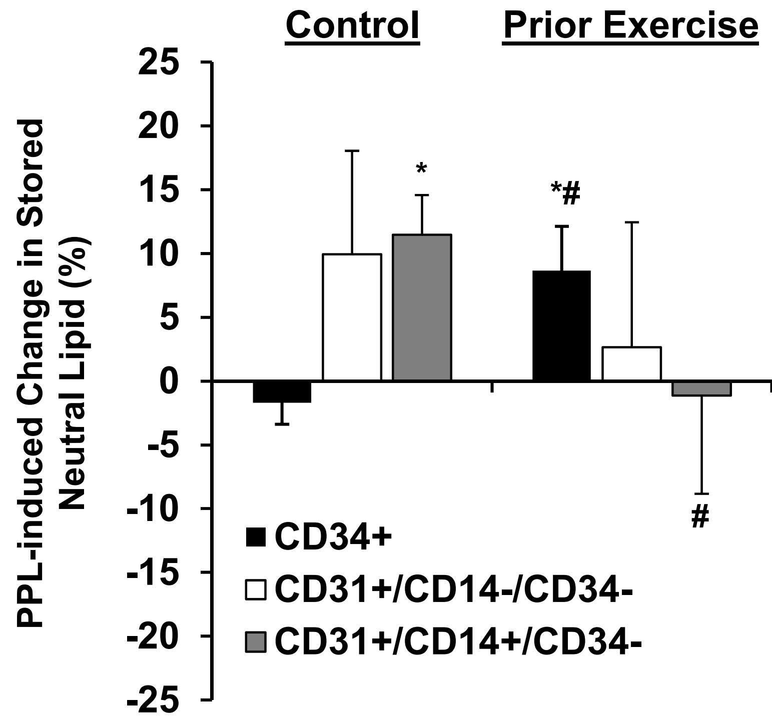


Figure 5.3. PPL-induced changes in stored neutral lipid in circulating angiogenic cells with and without prior endurance exercise. *Statistically significant change within trial (i.e., the percent change is different from zero; P < 0.05). #Magnitude of change is significantly different between trials (P < 0.05).

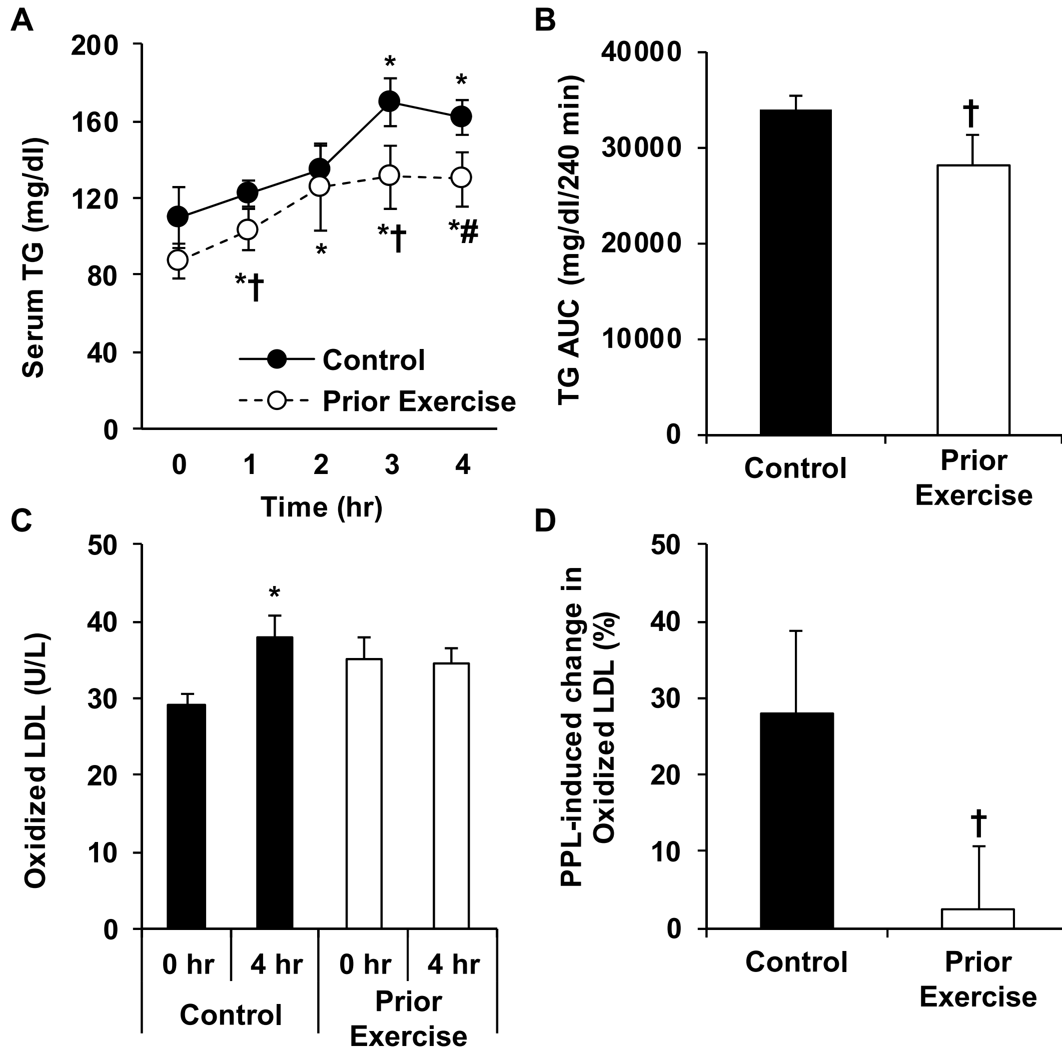


Figure 5.4. Effects prior exercise on PPL as measured by serum TG concentrations (A), TG AUC (B), and oxLDL (C). (D) PPL-induced change (%) in OxLDL in control and prior exercise trials. *Statistically significant difference from 0-hr time point within trial (P < 0.05). †Statistically significant difference between trials (P < 0.05). #P = 0.06 for difference between trials in serum TG concentration at 4-hr time point.

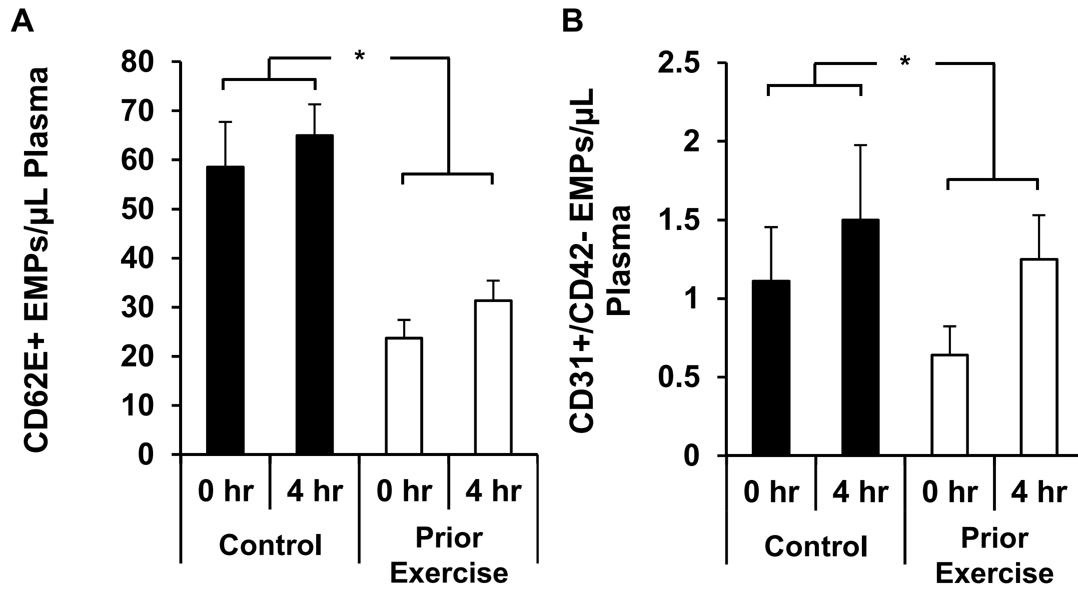


Figure 5.5. Effects of postprandial lipemia with and without prior endurance exercise on plasma endothelial microparticles (EMPs). (A) EMP fragments of activated endothelium (CD62E+ EMPs); (B) apoptotic endothelial CD31+/CD42- EMPs. *P < 0.05 for main effect of exercise.

Expanded Materials and Methods

Screening

Ten healthy, nonsmoking, recreationally- to highly-active (endurance exercise ≥4hr/week) men aged 18-35 yrs with no history of CV or metabolic disease were recruited for this study. Potential subjects were initially screened by telephone or email, and reported to the laboratory following an overnight fast for a screening visit to verify eligibility. Exclusion criteria were as follows: systolic blood pressure ≥130 mm Hg, diastolic blood pressure ≥90 mm Hg, serum total cholesterol level ≥200 mg/dl; low-density lipoprotein-cholesterol level of ≥130 mg/dl; high-density lipoprotein-cholesterol of ≤ 35; fasting glucose ≥100 mg/dl). The University of Maryland College Park Institutional Review Board approved all study procedures and subjects provided written informed consent.

A blood sample was obtained for assessment of fasting serum triglycerides (TG), lipoprotein lipids, and glucose (Quest Diagnostics, Baltimore, MD). Height, weight, and body mass index were measured, and body composition was assessed using the 7-site skinfold procedure (107). Maximal oxygen uptake ($\dot{V}O_2max$) was determined using a stationary cycling test to exhaustion (Life Cycle). Subjects began cycling at 200W, and the work rate increased by 25W every 2 min until the subject was unable to maintain a cycling cadence of ≥50 revolutions/min. $\dot{V}O_2$ was measured continuously via an automated indirect calorimetry system (Oxycon Pro). All subjects achieved a valid $\dot{V}O_2max$ as indicated by the plateau criteria (≤200 ml/min increase in $\dot{V}O_2$ with increase in work rate).

Experimental Protocol

For the exercise trial, subjects reported to the laboratory at 3:00 PM, and performed stationary cycling at 70% of $\dot{V}O_2max$ until a total energy expenditure of 2.5 MJ (598 kcal) was reached. This exercise protocol was chosen because exercise of this intensity and energy expenditure have been shown to consistently reduce PPL in response to high fat meals (164). Exercise intensity was verified by measurement of $\dot{V}O_2$ via indirect calorimetry every 10 min during exercise. The exact work rate and duration of exercise for each subject are presented in Supplemental Table 5.I. For the control trial, subjects remained sedentary for the 24 hours prior to the PPL test. Subjects were instructed to maintain the same dietary habits for 72 hr preceding each PPL test, which was verified by dietary logs.

Because the effect of exercise on PPL is influenced by the timing, caloric content, and composition of meals ingested following exercise (80), participants consumed a meal of fixed macronutrient composition (40% carbohydrate, 30% fat, and 30% protein) between 7:00 and 8:00 PM, as described previously (197). This meal consisted of commercially available Zone Perfect bars (Abbott Nutrition, Columbus, OH). The number of bars eaten by each participant was calculated and portioned by weight to provide 0.5 g of carbohydrate/kg BW and 20.9 kJ/kg BW. Subjects were asked to sleep for at least 8 hr prior to PPL testing.

PPL tests began at 6:00 AM on the morning following each treatment and were performed as described by our laboratory previously (247). The test meal contained heavy whipping cream, sugar, chocolate syrup, and nonfat powdered milk. The size of the test meal was normalized to each subject's body surface area (386 g/2 m^2 body surface area). A 386-g serving of the meal provided 1362 kcal (84% fat). The exact

meal composition for each subject is presented in Supplementary Table 5.II. All subjects consumed their test meals within 5 min and the meals were well-tolerated. Thirty-ml blood samples (K_2EDTA Vacutainer Tubes, Becton Dickinson) were obtained at baseline (0 min) and 240 min for isolation of $CD34^+$, $CD31^+/CD14^-/CD34^-$, and $CD31^+/CD14^+/CD34^-$ CACs. Plasma and serum samples were obtained before the meal and at 1, 2, 3, and 4 hr postprandial for assessment of serum triglyceride and oxidized LDL (OxLDL) concentrations and plasma endothelial microparticle (EMP) concentrations.

Immunomagnetic Cell Separation

Peripheral blood mononuclear cells (PBMCs) were isolated from 0- and 4-hr samples using density gradient centrifugation (Ficoll, GE Healthcare). The $CD34^+$, $CD31^+/CD14^-/CD34^-$, and $CD31^+/CD14^+/CD34^-$ fractions were purified using multiple rounds of immunomagnetic cell separation according to the manufacturer's instructions (EasySep® Immunomagnetic Cell Separation Kits, STEMCELL Technologies). In the first step, PBMCs were sorted into $CD34^+$ and $CD34^-$ fractions. The $CD34^-$ fraction was then sorted into $CD31^+/CD34^-$ and $CD31^-/CD34^-$. The $CD31^-/CD34^-$ fraction was discarded. In the final sorting step, the $CD31^+/CD34^-$ fraction was further divided into $CD14^+$ and $CD14^-$ fractions. Each of these sorting procedures was performed as follows: cells were resuspended in PBS + 2% FBS at a density of 2×10^8 cells/ml. The positive selection antibody cocktail was added (100 µl/ml) to the cell suspension and incubated at room temperature for 15 min. Magnetic microparticles were then added (100 µl/ml) and incubated for 10 min, and PBS + 2% FBS was added to bring suspensions to a final volume of 2.5 ml. Cells were then

incubated in the selection magnet for 5 min. Positively-selected cells remained attached to the side of the tube, and negatively-selected cells were poured off in the supernatant. The magnetic incubation step was repeated, and positive and negative cell fractions were counted by hemocytometer. The validity of the $CD34^+$ PBMC immunomagnetic selection procedure has been previously confirmed in our laboratory (108). For validation of the CD14 and CD31 selection procedures, $CD14^-$, $CD14^+$, $CD31^+$, and $CD31^-$ selected fractions were analyzed by RT-PCR. RNA was isolated and converted to cDNA as described below and cDNA was assessed for CD31 and CD14 gene products using PCR and followed by electrophoresis on ethidium bromide-stained 1.5% agarose gels (CD31 forward primer: 5'-CCCAGGAGCACCTCCAGCC-3'; CD31 reverse primer: GGACCTCATCCACCGGGGCT-3'; CD14 forward primer: 5'-GGGCGCCTGAGTCATCAGGACAC-3'; CD14 reverse primer: 5'-CAAGGTTCTGGCGTGGTCGCA-3'). 18S was used as the reference gene. 18S primer sequences were published previously (109). For both selection antibodies, positively-selected cells displayed strong expression of the target antigens, while CD31 and CD14 mRNAs were not detectable in the negatively-selected fractions (Supplemental Figure 5.I).

Pharmacological Treatments and Measurement of Intracellular NO and ROS

These experiments were performed in duplicate as we have described previously (108, 109), with modifications to examine freshly-isolated cells in a 96-well format. Briefly, 1.5×10^5 cells stained with 10 μM DAF-FM diacetate for determination of intracellular NO levels or 2 μM 2′,7′-dichlorodihydrofluorescein diacetate

(H_2DCFDA) for determination of intracellular ROS levels (Molecular Probes). DAPI (750 ng/ml) was used to label cell nuclei (Molecular Probes). Cells were also incubated with or without 250 μM apocynin (a pharmacologic NADPH oxidase inhibitor) and/or 1 μM rotenone (an inhibitor of mitochondrial complex I) to determine the contribution of NADPH oxidase- and mitochondrial-derived ROS to any observed effects of PPL and/or exercise on intracellular ROS and NO levels. Rotenone treatments were performed on cells from a subset of subjects (n = 5), and, owing to limiting cell yields, were only performed on cells that were analyzed for intracellular ROS levels. Cells were incubated with fluorescent dyes and drug or vehicle treatments in a final volume of 150 μl serum-free PBS for 60 min at 37°C. Plates were then centrifuged at 500*g* for 5 min and cells were resuspended in 150 μl PBS. NO and ROS fluorescence were quantified using a fluorescent plate reader (Wallac Victor^2 1400, Perkin Elmer) using excitation and emission filters of 488 and 535 nm, respectively. DAPI fluorescence was measured using excitation and emission filters of 355 and 460 nm, respectively. NO and ROS fluorescence values were divided by DAPI fluorescence values for normalization to cell number, and data are expressed relative to the mean for the no exercise 0-min value. Intra-assay coefficients of variation for NO and ROS were both 5%. Because each assay was performed on different days for each subject, inter-assay coefficients of variation were determined on blank samples (PBS only) to document the day-to-day variability in arbitrary/background fluorescence. The inter-assay coefficients of variation were 5% and 4% for NO and ROS assays, respectively, indicating good day-to-day reliability of the assays. For validation of the DAF fluorescent probe, unfractionated

PBMCs and the immunomagnetically-selected CAC populations (n = 3 pilot experiments) were treated with a NOS inhibitor (L-NAME) and the NO donor, (3-morpholino-sydnomine). L-NAME reduced DAF fluorescence signals to ~10-20% of basal levels, while 3-morpholino-sydnomine dose-dependently increased the DAF signal by ~1200-1800 fold compared to basal levels. Similarly, for validation of the ROS probe H_2DCFDA, the SOD mimetic reduced DCF fluorescence by >50% in all cell populations, whereas 3-morpholino-sydnomine (also ROS donor) increased DCF fluorescence by ~1000 fold compared to basal levels.

Intracellular Lipid Assay

In cells from a subset of subjects (n = 5), intracellular lipids were measured with 1 μM BODIPY 493/503 (Molecular Probes), a stain for intracellular nonpolar lipids, using excitation and emission filters of 488 and 535 nm, respectively. Stored neutral lipid fluorescence values were divided by DAPI fluorescence for normalization to cell number. Data are expressed as percent change from 0 to 240 min within each experimental condition.

Assessment of Gene Expression by RT-PCR

RNA was isolated using the TRI reagent and reversed transcribed to cDNA. mRNA levels of endothelial/angiogenic [endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF)], pro-oxidant [inducible NOS (iNOS), oxLDL receptor (LOX-1), and NADPH oxidase subunits $gp91^{phox}$ and $p47^{phox}$], and antioxidant genes (SOD1 and SOD2) were assessed using semiquantitative RT-PCR. eNOS, gp91phox, p47phox, SOD1, and SOD2 primer sequences are published elsewhere (109). Additional gene targets included VEGF (forward primer: 5'-

AAGGAGGAGGGCAGAATCAT-3’, reverse primer: 5’-ATCTGCATGGTGATGTTGGA-3’), iNOS (forward primer: 5’-GGCCGCAGAGAACTCAGCCTCA-3’, reverse primer: 5’-CTCAAAACAGCCGCTTCCCCAGAA-3’), and the oxLDL receptor-1 (LOX-1; forward primer: 5’-TTACTCTCCATGGTGGTGCC-3’, reverse primer: 5’-AGCTTCTTCTGCTTGTTGCC-3’). Gene products were amplified using optimized thermal cycling conditions followed by electrophoresis on ethidium bromide-stained 1.5% agarose gels. For each target gene, all samples within each cell type were run on the same gel. Gels were visualized under UV light, and band intensities were quantified using ImageJ. Data were normalized to the reference gene 18S, and are presented relative to the normalized value for the control 0-hr condition (set at 100%).

Serum Triglyceride and Oxidized LDL-cholesterol Concentrations

Serum TG concentrations were examined using a standard colorimetric assay (Sigma), as described by our laboratory previously (247). Total areas under the TG curves (TG AUC) were calculated using the trapezoidal principle (140). The TG intra-assay coefficient of variation was 6.5%, and the inter-assay coefficient of variation was 8.5%. Serum levels of oxidized LDL cholesterol (OxLDL) were determined using a sandwich ELISA (Mercodia) according to the manufacturer’s instructions. Serum OxLDL was measured at 0- and 4-hr only. The OxLDL assay intra-assay coefficient of variation was 4%. All samples were analyzed in the same batch to eliminate inter-assay variability.

Plasma Endothelial Microparticles (EMPs)

Preparation of Plasma: Plasma samples were thawed at room temperature and centrifuge at 1500*g* x for 20 minutes at room temperature to obtain platelet poor plasma (PPP), The top 2/3 volume of PPP was further centrifuged at 1500*g* for 20 minutes at room temperature to obtain cell free plasma (CFP) (194). The supernatant was used for microparticle analysis (58). A volume of 100 µl supernatant was incubated with the different fluorochrome-labeled antibodies for 20 minutes at room temperature in the dark. Two different antibody combinations were used: CD31-phycoerythrin (PE, 20 µl/ sample) with CD42b-fluorescein isothiocyanate (FITC, 20 µl/sample), CD62E-PE (15 µl/sample). All antibodies were obtained from BD Biosciences. Samples were diluted with 500 ml of 0.22 µm double -filtered PBS before flow cytometric analysis.

Detection of endothelial microparticles using flow cytometry: Samples were analyzed using a BD LSRII flow cytometer and BD FACSDIVA software. EMPs were defined as CD31+CD42b- or CD62E+ events smaller than 1.0 µm. A logarithmic scale was implemented for forward scatter signal, side scatter signal and each fluorescent channel; size calibration was done with 0.9 µm standard precision NIST Traceable polystyrene particle beads (Polysciences, Inc.) and forward scatter signal. Fluorescence minus one (FMO) controls and non-stained samples were used to discriminate true events from noise, and to increase the specificity for microparticle detection for each sample. The flow rate was set on medium on LSRII and all samples were run for 180 seconds. Using beads, we calculated that, on medium flow rate, a mean sample volume of 101 µl/ 180 seconds was processed. EMP counts per µl plasma were determined using the following formula:

(Example for $CD31^+/CD42^-$ events):

$$\frac{\textbf{Number of CD31}^+\textbf{/CD42}^-\textbf{ events}}{\textbf{Volume sample analyzed}} \times \frac{\textbf{Total volume of sample}}{\textbf{Amount of PPP}}$$

Where: the total volume of the sample equals 100 µl PPP stained with 20 µl CD42b-FITC and 20 µl of CD31-PE diluted with 500 µl PBS, 93 µl formaldehyde; (733 µl for CD31+ CD42- and 713 µl for CD 62E), the volume of the sample analyzed by the flow cytometer in 180 seconds equals 101 µl; and the amount of PPP used for the analysis is 100 µl (232).

<u>Supplemental Results – RT-PCR</u>

In $CD31^+/CD14^-/CD34^+$ cells, eNOS mRNA levels were higher in the trial with exercise compared to control ($P < 0.05$), but were unaffected by PPL during either trial (Supplemental Fig 5.IIA). iNOS mRNA was unaffected by PPL in the control trial, but increased significantly with PPL in the trial with prior exercise ($P < 0.05$) such that iNOS levels were higher at the 4 hr PPL time point during the prior exercise trial compared to the 4 hr PPL time point of the control trial ($P < 0.05$; Fig 5.IIB). VEGF mRNA levels significantly decreased with PPL during the control trial ($P < 0.05$), yet increased significantly during the prior exercise trial ($P < 0.05$; Fig 5.IIC). SOD1 mRNA increased with PPL during the control trial ($P < 0.05$; Fig 5.IID), but there was no effect of PPL in the prior exercise trial (Fig 5.IID). There were no effects of PPL or differences between trials for SOD2 or $gp91^{phox}$ mRNA levels (Fig 5.IIE-F). $p47^{phox}$ and LOX-1 (Fig 5.IIG-H) mRNA levels displayed similar patterns in response to prior exercise and PPL, with no effects of PPL in the control trial,

although both of these genes were significantly increased in the trial with prior exercise ($P < 0.05$).

In $CD31^+/CD14^+/CD34^-$ cells, PPL increased eNOS mRNA in the control trial ($P < 0.05$) but there was no change in the prior exercise trial (Fig 5.IIIA). There were no changes in iNOS mRNA with PPL during either trial (Fig 5.IIIB). VEGF mRNA was significantly reduced at 0 hr in the prior exercise trial compared to 0 hr in the control trial ($P < 0.05$), and tended to increase ($P = 0.07$) in the prior exercise trial (Fig 5.IIIC). There were no effects of PPL during either trial on SOD1 or SOD2 mRNA levels (Fig 5.IIID-E). $gp91^{phox}$ mRNA did not change significantly with PPL in the control but increased significantly in the trial with prior exercise ($P < 0.05$; Fig 5.IIIF). PPL induced significant increases in $p47^{phox}$ mRNA in both trials ($P < 0.05$; Fig 5.IIIG). LOX-1 mRNA levels were higher at both time points of the prior exercise trial compared to the control trial ($P < 0.05$) but were unaffected by PPL in both trials (Fig 5.IIIH).

In $CD34^+$ cells, eNOS mRNA increased significantly with PPL in the control trial but there was no change in the prior exercise trial (Supplemental Fig 5.IVA). There were no effects of exercise or lipemia on iNOS, VEGF, SOD1, SOD2, or $gp91^{phox}$ mRNA ($P > 0.05$; Fig 5.IVB-F). $p47^{phox}$ increased significantly with PPL during both the control and prior exercise trials ($P < 0.05$; Fig 5.IVG). LOX1 mRNA was unaffected by prior exercise or PPL (Fig 5.IVH).

Supplemental Table 5.I. Duration of submaximal exercise at 70% $\dot{V}O_2$max

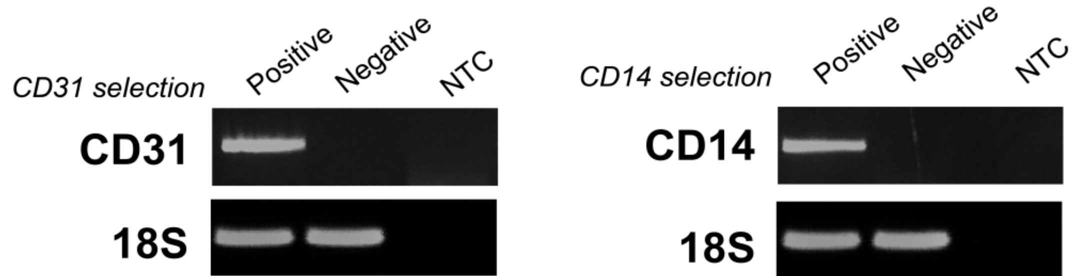
ID	Body mass (kg)	$\dot{V}O_2$max (ml/kg/min)	70% $\dot{V}O_2$max (ml/kg/min)	70% $\dot{V}O_2$max (L/min)	Work rate (Watts)*	Duration (min)**
01	78.4	50.0	35.0	2.74	203	43.5
02	63.4	61.1	42.8	2.71	210	44.1
03	72.6	54.5	38.2	2.77	209	43.2
04	97.0	50.5	35.4	3.43	255	34.9
05	64.0	42.1	29.5	1.89	133	63.3
06	87.5	52.2	36.5	3.20	239	37.4
07	83.2	47.0	32.9	2.74	199	43.7
08	87.5	41.6	29.1	2.55	179	46.9
09	81.1	42.0	29.4	2.38	168	50.1
10	68.6	38.9	27.2	1.87	129	63.9
Mean	78.3	48.0	33.6	2.6	193	47.1
SD	11.1	7.0	4.9	0.5	41	9.7
SEM	3.5	2.2	1.5	0.2	13	3.1

*Calculated using the American College of Sports Medicine for $\dot{V}O_2$ during cycle ergometry (6): $\dot{V}O_2$ = Watts/Mass × 10.8 + 7.

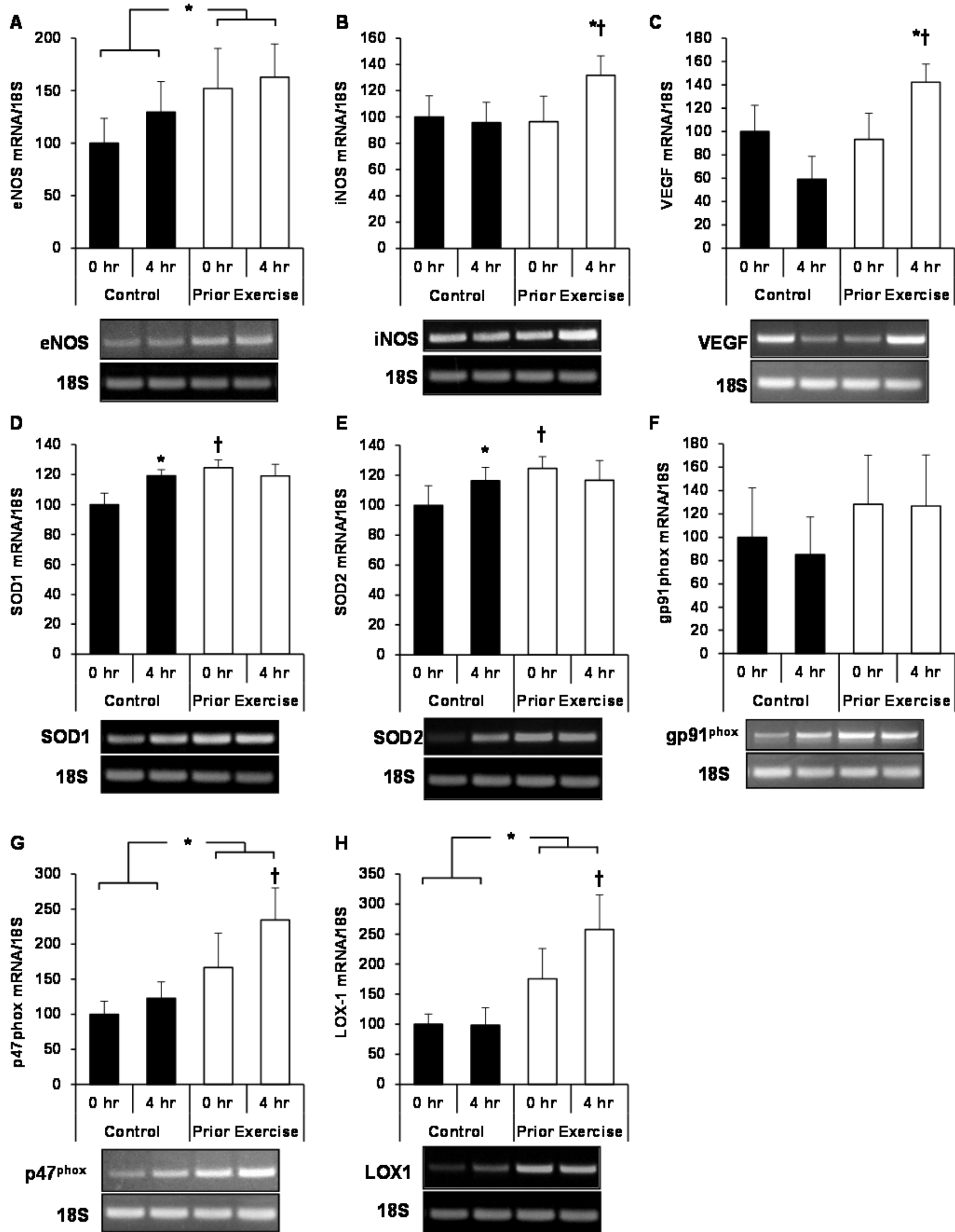
**Time required to reach an energy expenditure of 2.5 MJ (598 kcal) using assumption that 1 L O2 consumed = 5 kcal energy expenditure.

Supplemental Table 5.II. PPL test meal composition for each subject.

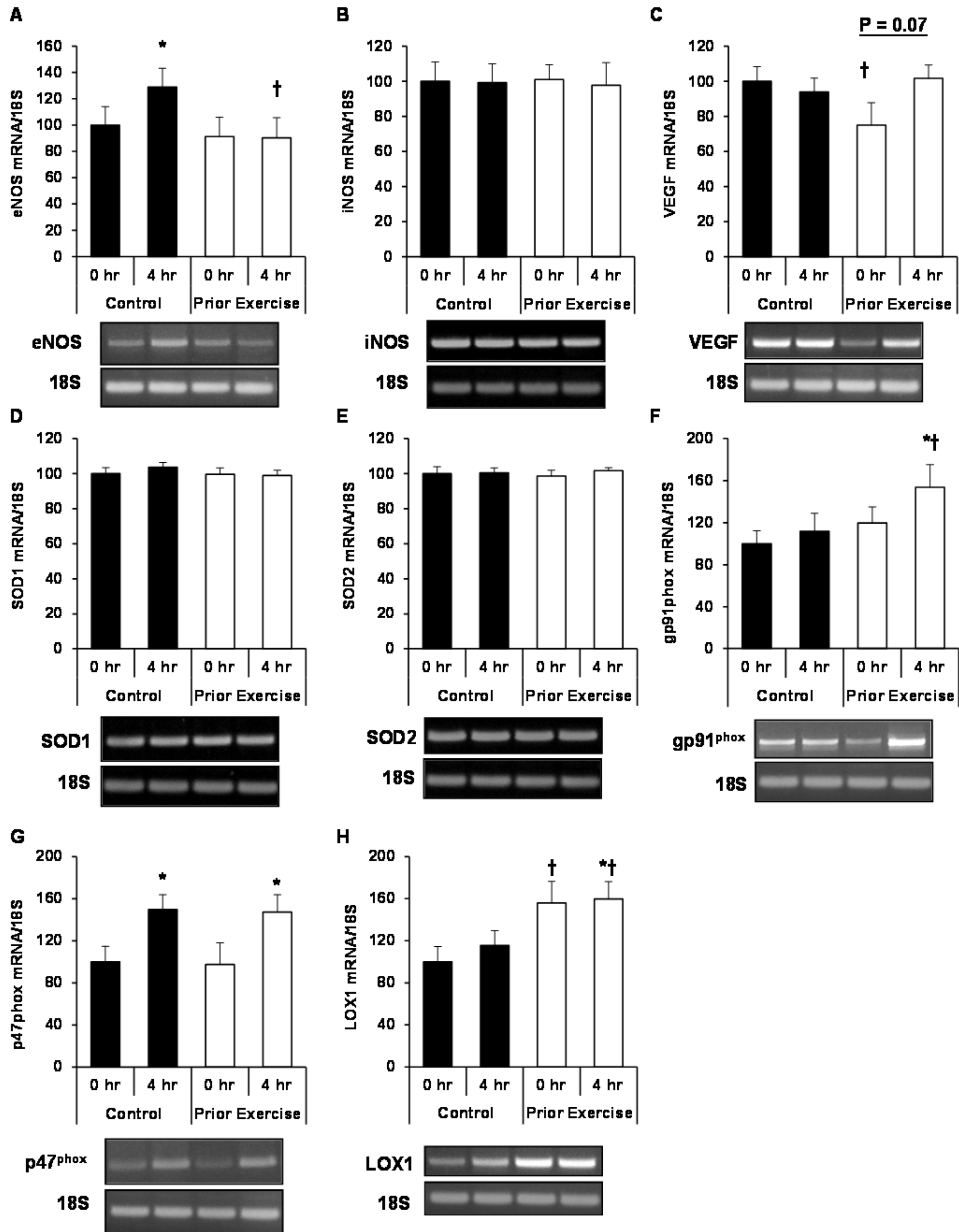
ID	Weight (lbs)	Height (inches)	Body Surface Area (m^2)	Meal Volume (mL)	Meal Volume (fl oz)	Meal Calories (kcal)	Protein Calories (kcal)	CHO Calories (kcal)	Fat Calories (kcal)
01	172.5	69	1.91	364	12.3	1303	36	182	1121
02	139.5	67	1.71	325	11.0	1164	33	163	1001
03	159.7	69	1.84	350	11.8	1254	35	176	1078
04	213.4	72	2.16	411	13.9	1472	41	206	1266
05	140.9	66	1.70	323	10.9	1157	32	162	995
06	192.5	71	2.05	389	13.1	1394	39	195	1199
07	183.0	73	2.05	389	13.1	1393	39	195	1198
08	192.5	73	2.08	395	13.4	1417	40	198	1218
09	172.5	69	1.91	364	12.3	1303	36	182	1121
10	151.0	70	1.82	346	11.7	1241	35	174	1067
Mean	172	70	2	365	12	1310	37	183	1126
SD	24	2	0.2	30.1	1.0	108	3	15	93
SEM	8	1	0.05	9.5	0.3	34	1	5	29



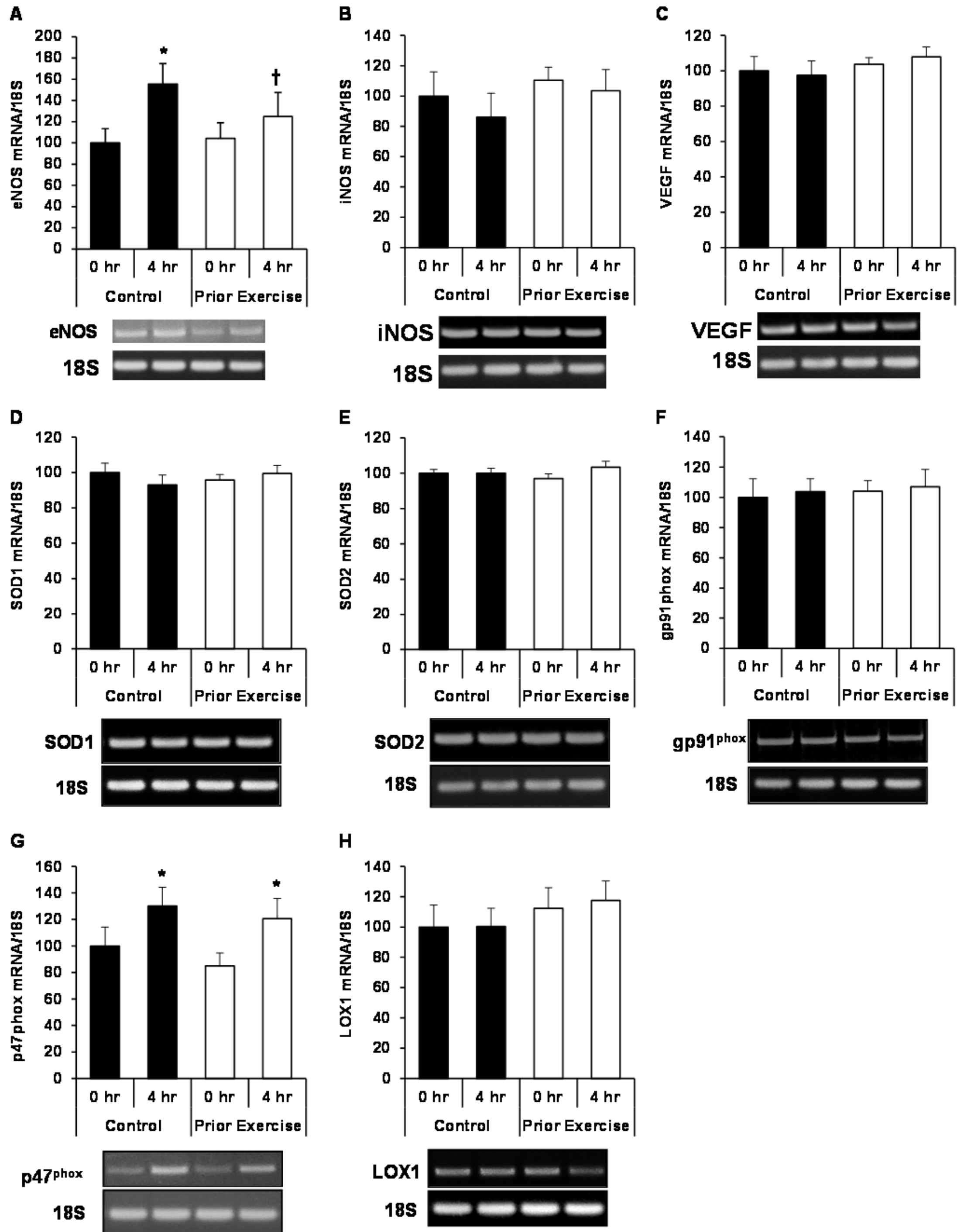
Supplemental Figure 5.I. Validation of immunomagnetic separation of $CD31^+$ (left) and $CD14^+$ (right) peripheral blood mononuclear cells (PBMCs) by RT-PCR. NTC: no template control.



Supplemental Figure 5.II. Effects of postprandial lipemia with and without prior endurance exercise on eNOS (A), iNOS (B), VEGF (C), SOD1 (D), SOD2 (E), gp91^{phox} (F), p47^{phox} (G), and LOX1 (H) mRNA levels in CD31+/CD14-/CD34- cells. *Statistically significant change from within-trial 0 hr sample ($P < 0.05$). †Statistically significant difference from corresponding time point between trials ($P < 0.05$).



Supplemental Figure 5.III. Effects of postprandial lipemia (PPL) with and without prior endurance exercise on eNOS (A), iNOS (B), VEGF (C), SOD1 (D), SOD2 (E), gp91^{phox} (F), p47^{phox} (G), and LOX1 (H) mRNA levels in CD31+/CD14+/CD34- cells. *Statistically significant change from within-trial 0 hr sample ($P < 0.05$). †Statistically significant difference from corresponding time point between trials ($P < 0.05$).



Supplemental Figure 5.IV. Effects of postprandial lipemia with and without prior endurance exercise on eNOS (A), iNOS (B), VEGF (C), SOD1 (D), SOD2 (E), gp91^{phox} (F), p47^{phox} (G), and LOX1 (H) mRNA levels in CD34+ cells. *Statistically significant change from within-trial 0 hr sample ($P < 0.05$). †Statistically significant difference from corresponding time point between trials ($P < 0.05$).

Chapter 6: Summary and Conclusions

Summary

The three studies performed for this dissertation have contributed the following new information: (i) regular endurance exercise is associated with greater intracellular NO in cultured CACs, partly due to physical activity-dependent down-regulation of NADPH oxidase activity (study #1), (ii) a sedentary lifestyle is associated with nitro-oxidative stress in freshly isolated $CD34^+$ progenitor cells (study #2), and (iii) prior endurance exercise prevents PPL-induced increases in ROS production in $CD31^+$ CACs (study #3). With a few notable exceptions (discussed in the Discussion section of each manuscript), it can be concluded that, in general, the hypotheses outlined in Chapter 1 were supported by these findings. Although these studies provide some of the first information about the effects of acute and chronic endurance exercise on molecular regulators of CAC function, a number of important questions remain for future research.

Perspectives for Future Research

Is NO a valid surrogate marker for CAC functional capacity? Substantial evidence from the literature indicates that exercise training can improve CAC function as indicated by migratory capacity and reendothelialization assays (93, 198, 228, 230). It is also generally thought that these functions of CAC functions are dependent on the availability and actions of NO (61, 250). However, the present data (from study #2) indicating an inverse relationship between exercise training status and intracellular NO levels in $CD34^+$ cells would suggest that NO may not be an all-encompassing indicator of the functional status of these cells. Based on the vast

amount of information relative to the beneficial health consequences of exercise, it is unlikely that the lower NO levels in CD34$^+$ cells from the trained group should be taken as an indicator that these cells were dysfunctional. Rather, the discrepancy probably reflects the dual role that NO plays in cells of the immune system. NO is elevated in nitro-oxidative stress and, in high concentrations, can play a role in inflammatory responses (159, 213). As discussed in Chapter 4, it is likely that the higher NO levels (combined with higher $O_2^{\cdot-}$, higher iNOS mRNA levels, and lower eNOS mRNA levels) in CD34$^+$ cells from the sedentary group is suggests a pro-inflammatory/pro-oxidative cell phenotype associated with a sedentary lifestyle. Although this conclusion was somewhat speculative, there is substantial evidence that sedentary behavior increases systemic inflammation and oxidative stress (163). Unfortunately, it was not possible to prove that the higher NO in cells from sedentary individuals in study #2 was linked to chronic inflammation/oxidative stress given the cross-sectional study design. Also, it would have been advantageous to have had a measure of angiogenic capacity (e.g., in vitro vessel formation, which was not performed because of limiting cell yields), as such data would have been helpful in interpreting whether higher NO levels were associated with impaired CAC function. Nevertheless, it is reasonable to suggest that the role of NO as a marker of CAC functional capacity needs to be re-evaluated. Future exercise studies must link measures of NO and ROS to measures of CAC function to definitively clarify the mechanistic role of NO-ROS dynamics in exercise-induced alterations in CAC-mediated effects on the vascular endothelium.

Are CACs required for exercise training-induced effects on the vasculature? The present studies focused on genes (antioxidant defenses, pro-oxidant enzymes, and regulators of angiogenesis) and intracellular molecules (NO and ROS) that had previously been implicated in functional activities of CACs. However, these experiments may have been somewhat premature, because at this point we still do not know whether or to what extent CACs are actually involved in exercise training effects on the vascular endothelium. Probably because of technical limitations, no studies have examined the effect of *in vivo* CAC depletion on exercise training-induced angiogenesis or effects on endothelial function. However, pharmacologic manipulation of circulating monocyte concentrations in rodents indicated that VEGF- and ischemia-induced angiogenesis is impaired in the presence of low circulating monocyte levels (86). Clearly a similar approach applied to an exercise training model would provide critical information to form appropriate hypotheses dealing with the effects of acute exercise or exercise training on CAC number and function. Thus, although we know that regular exercise can prevent or delay the onset of endothelial dysfunction and CV disease, or even reverse existing CV pathologies, future work must determine whether and quantify the extent to which CACs are involved in mediating the effects exercise training on CV health.

Cell therapy: the future of exercise-based CAC research? While the present research investigations were underway, a substantial number of papers were published on the use of CACs as a source for cell therapeutic treatment of ischemic CV diseases. A recent review of 19 published cell therapy studies and 31 registered ongoing clinical trials concluded that although the prospect of "regenerative

medicine" using cell therapy is promising, the results are too variable for widespread clinical use (156). Current strategies to improve the efficacy of cell therapy include the use of mobilizing agents, pharmacologic adjuvants, *ex vivo* CAC expansion, and gene modification (156). One strategy conspicuously absent from this list is exercise, and, indeed, our laboratory has recently proposed that exercise may be one way to improve CAC-mediated regenerative angiogenesis (250). Specifically relating to the results of the present studies, the following hypotheses are proposed. Based on study #1, preconditioning patients with a bout of acute exercise prior to *ex vivo* expansion in culture may reduce oxidative stress and increase NO bioavailability of CACs to be used for therapy. From study #2, it might be expected that an exercise training intervention prior to CAC infusions would reduce nitro-oxidative stress and increase the expression of genes involved in angiogenesis in $CD34^+$ progenitor cells, a cell population that was recently shown to improve left ventricular function after coronary artery infusion in post-myocardial infarction patients (171). Finally, the results of study #3 would suggest that exercise might improve the ability of $CD31^+$ cells, recently shown to be effective source of cell therapy in rodent models of ischemic CV disease (114, 115), to better cope with an inflammatory/pro-oxidative challenge and thereby result in improved therapeutic outcomes.

Conclusions

Overall, it is concluded that acute and chronic endurance exercise enhance intracellular NO and ROS dynamics in CACs. The nature and direction of these effects appear to vary according to methodological approaches, such as cultured vs. freshly-isolated CACs and choice of cell surface antigens used to identify CAC

subpopulations. The roles of NO and ROS in CAC function and the extent to which CACs contribute to exercise training-induced improvements in vascular endothelial function will require clarification in future studies. Finally, the present studies may provide useful information for the development of future examinations of whether exercise can enhance the therapeutic efficacy of CACs in the treatment of CV disease.

Appendices

Appendix A - Approval Letters from the Institutional Review Board of the University of Maryland, College Park.

March 12, 2008

To: **Investigator:** James M. Hagberg
Co-Investigator(s): Not Applicable
Student Investigator(s): Sarah Witkowski
Department: KNES - Kinesiology

From: Roslyn Edson
IRB Manager
University of Maryland, College Park

Re: IRB Application Number 06-0447 (PAS# 1486.5)
Life-Long Exercise and Novel Cardiovascular and Aging Risk Factors

Approval Date: 02-21-2008

Expiration Date: 10-18-2008

Type of Application: Addendum (To include Nathan Jenkins as a student investigator on the project, add two additional younger groups of 12 healthy male participants between 18 and 30 years of age, recruit the UMCP varsity-and-club-level crosscountry and track teams and if necessary, via personal contacts with local running/cycling teams and clubs, draw the same amount of blood from the two groups of younger individuals as approved for the older participants, omit the forearm blood flow and the body composition assessment via DEXA procedures and access body composition using the skinfold technique. Also, a physician will no longer supervise the maximal exercise testing of the trained and untrained younger subjects.)

Type of Research: Non-Exempt

Type of Review: Expedited

The University of Maryland, College Park Institutional Review Board (IRB) approved your IRB application. The research was approved in accordance with the University's IRB policies and procedures and 45 CFR 46, the Federal Policy for the Protection of Human Subjects. Please include the above-cited IRB application number in any future communications with our office regarding this research.

Recruitment/Consent: For research requiring written informed consent, the IRB-approved and stamped informed consent document is enclosed. The IRB approval expiration date has been stamped on the informed consent document. Please keep copies

of the consent forms used for this research for three years after the completion of the research.

Continuing Review: If you want to continue to collect data from human subjects or analyze data from human subjects after the expiration date for this approval, you must submit a renewal application to the IRB Office at least 30 days before the approval expiration date.

Modifications: Any changes to the approved protocol must be approved by the IRB before the change is implemented except when a change is necessary to eliminate apparent immediate hazards to the subjects. If you want to modify the approved protocol, please submit an IRB addendum application to the IRB Office.

Unanticipated Problems Involving Risks: You must promptly report any unanticipated problems involving risks to subjects or others to the IRB Manager at 301-405-0678 or redson@umresearch.umd.edu.

Student Researchers: Unless otherwise requested, this IRB approval document was sent to the Principal Investigator (PI). The PI should pass on the approval document or a copy to the student researchers. This IRB approval document may be a requirement for student researchers applying for graduation. The IRB may not be able to provide copies of the approval documents if several years have passed since the date of the original approval.

Additional Information: Please contact the IRB Office at 301-405-4212 if you have any IRB-related questions or concerns.

August 27, 2010

To: **Investigator:** James M. Hagberg
Co-Investigator(s): Not Applicable
Student Investigator: Nathan Thomas Jenkins
Department: KNES - Kinesiology

From: Joseph M. Smith, MA, CIM
Manager
University of Maryland, College Park

Re: **IRB Application Number:** 10-0481 (PAS# 3162)
Project Title: "Effect of endurance exercise on angiogenic cells and growth factors during postprandial lipemia."

Approval Date: 08-23-2010

Expiration Date: 08-23-2011

Type of Application: New Application

Type of Research: Non-Exempt

Type of Review: Expedited

The University of Maryland, College Park Institutional Review Board (IRB) approved your IRB application. The research was approved in accordance with the University's IRB policies and procedures and 45 CFR 46, the Federal Policy for the Protection of Human Subjects. Please reference the above-cited IRB application number in any future communications with our office regarding this research.

Recruitment/Consent: For research requiring written informed consent, the IRB-approved and stamped informed consent document is enclosed. The IRB approval expiration date has been stamped on the informed consent document. Please keep copies of the consent forms used for this research for three years after the completion of the research.

Continuing Review: If you want to continue to collect data from human subjects or analyze data from human subjects after the expiration date for this approval, you must submit a renewal application to the IRB Office at least 30 days before the approval expiration date.

Modifications: Any changes to the approved protocol must be approved by the IRB before the change is implemented except when a change is necessary to eliminate apparent immediate hazards to the subjects. If you want to modify the approved protocol, please submit an IRB addendum application to the IRB Office.

Unanticipated Problems Involving Risks: You must promptly report any unanticipated problems involving risks to subjects or others to the IRB Manager at 301-405-0678 or jsmith@umresearch.umd.edu.

Student Researchers: Unless otherwise requested, this IRB approval document was sent to the Principal Investigator (PI). The PI should pass on the approval document or a copy to the student researchers. This IRB approval document may be a requirement for student researchers applying for graduation. The IRB may not be able to provide copies of the approval documents if several years have passed since the date of the original approval.

Additional Information: Please contact the IRB Office at 301-405-4212 if you have any IRB-related questions or concerns.

Appendix B – IRB-approved Informed Consent Forms.

Project Title: **Life-Long Exercise and Novel Cardiovascular Disease and Aging Risk Factors – Young Active Participants**

CONSENT FORM

This is a research project being conducted by Dr. James Hagberg in the Department of Kinesiology at the University of Maryland College Park. We are inviting you to participate in this research project because you are 18 – 35 years of age, are generally healthy, and have a long history of exercise/physical activity. The purpose of this study is to determine whether aging and regular exercise are associated with novel risk factors related to cardiovascular disease. In this case, "novel" risk factors mean newer risk factors beyond the standard risk factors such as cholesterol levels, blood pressure, diabetes, and obesity. In a subset of exercisers we will determine the degree to which these risk factors change when they stop exercising for 10 days. Two groups of older participants, one consisting of life-long exercisers and the other of generally sedentary individuals, have already completed the study. We recruit you for the study because we would like to compare the levels of novel risk factors from these older groups to those from younger individuals who exercise regularly. Your participation in this research is completely voluntary and you may choose not to take part at all. You are free to ask questions at any time without penalty. If you decide to participate in this research, you may stop participating at any time. If you decide not to participate in this project or if you stop participating at any time, you will not be penalized or lose any benefits to which you otherwise qualify. The specific tests, their requirements, and time commitments are described below.

Participant Characteristics: You have already completed a telephone or personal interview that you verbally consented to that determined that you are 18 – 35 years of age, regularly engage in endurance exercise such as running or cycling, are not a diabetic, have no evidence of cardiovascular or lung disease, and have no other medical problems that keep you from exercising vigorously. We have also discussed with you that certain medications may exclude you from taking part in this study.

Study Procedures: If you qualify and complete the phase of this study where you stop exercising for 10 days, your total involvement in this study will consist of 3 visits over 2 – 3 wks. If you do not undergo the 10 days of stopping exercise, your total involvement will consist of 2 visits over approximately 1 week.

Screening Visit: For the Screening Visit, the study will be explained to you, your medical history will be reviewed, and you will be asked to provide your written informed consent. Your resting blood pressure will be measured and about 2 tablespoons of blood will be drawn from a vein in your arm. Your body composition will be assessed by a procedure that involves measuring the thickness of your skinfolds. You will then undergo a treadmill exercise test to determine your maximal exercise capacity. This test will be done on an exercise treadmill where the treadmill incline will increase every 2 minutes until you cannot continue. Blood pressure, heart rate, and the electrical activity of your heart (electrocardiogram) will be recorded before, during, and after the test. During this test you will have a noseclip on your nose and you will breathe through a mouthpiece so that the air that you breathe out can be analyzed. This visit will last about 1 hour.

 Date ______________ Initials ____________

Testing Visit #1: For the first Testing Visit, you will report to the laboratory in the morning after an overnight fast (only taking in water from 8 PM the night before). Prior to this visit you will record all items that you eat over a 7 day period. About 4 tablespoons of blood will be drawn from a vein in your arm. You will then undergo treadmill exercise for 30 minutes at a moderate to vigorous intensity. Heart rate and blood pressure will be monitored before, during, and after the exercise. During this test you will have a noseclip on your nose and you will breathe through a mouthpiece so that the air that you breathe out can be analyzed. At 5 and 30 minutes after the exercise approximately 4 tablespoons of blood will be drawn from a vein in your arm. The blood samples that you provide before and after exercise will be tested for levels of the new cardiovascular disease risk factors. This visit will last about 1.5 hours.

If you are not undergoing the 10 days without exercise, this is all the testing that is required of you. If you are undergoing the 10 days without exercise phase of the study, you will undergo 1 additional visit to the laboratory.

Testing Visit #2: Following 10 days of training cessation, you will report to the laboratory for a blood draw and a second assessment of your body composition. During the period of training cessation, we ask that you monitor your weight to ensure that you do not gain or lose any weight when you are not exercising. If you change any of your medications during this phase of the study, please notify one of the researchers. This visit will last about 30 minutes.

The maximum total amount of blood that will be drawn during the testing is about 12 tablespoons if you are not undergoing the 10 days without exercise and about 18 tablespoons over the space of 2 – 3 weeks if you are undergoing the 10 days without exercise. This is approximately 1/3 and 1/2, respectively, of the amount of blood that is typically drawn during a single blood donation. These samples will be used to measure a number of chemicals and cell numbers in the blood that may be related to a person's risk of cardiovascular disease and other age-related diseases.

☐ Yes, I consent to take part in Testing Visit #2
☐ No, I do not consent to take part in Testing Visit #2

☐ Yes, I consent to having my blood drawn and saved for future analyses
☐ No, I do not consent to having my blood drawn and saved for future analyses and my samples must be destroyed after the completion of this study.

Confidentiality: We will do our best to keep your personal information confidential. To help protect your confidentiality, all data are kept in a locked office with access available only to study personnel. Furthermore, all computer data bases containing results from this study will not have any names attached to them. If we write a report or article about this research project, your identity will be protected to the maximum extent possible. Your information may be shared with representatives of the University of Maryland College Park or government authorities if you or someone else is in danger or if we are required to do so by law.

Samples of your blood will be sent to other collaborating laboratories for other blood measurements. Your blood samples sent to these laboratories will be identified only by a numeric code and only investigators at the University of Maryland College Park will know whose name is associated with each coded number. The list of names and codes will be retained at the University of Maryland College Park for up to 25 years.

Risks: The following risks are associated with your participation in this study. (1) There is some risk associated with maximal exercise testing but complications are unusual in healthy individuals in your age group (Gibbons et al., 1989, *Circulation* Vol. 80, No. 4). Risks will be minimized by having the test administered by personnel trained in such tests and emergency procedures. You will gradually cool down upon reaching your highest effort, which will further help to reduce the risk of complications. A cardiac defibrillator will be present at all testing sessions. (2) There are risks of bruising and infection associated with blood drawing. These risks will be minimized by using sterile techniques and by having experienced personnel draw all blood samples. (3) The skinfold body composition testing has no known risks. (4) The risk of stopping your exercise for 10 days, if you complete this portion of the study, is that your risk factors for cardiovascular disease may deteriorate. It is unlikely that substantial changes will take place over the space of the 10 days without exercise. Furthermore, you will be able to start exercising again immediately after these 10 days without exercise. (5) There are no known risks associated with completing 7 day dietary records. (6) Your involvement will require some amount of time, travel, and effort. Your parking costs will be covered.

Benefits: This study may not help you personally, but may help the investigators to determine whether regular exercise results in better levels of new cardiovascular disease risk factors. Because you are a young and healthy participant, and you are therefore not at elevated risk for cardiovascular disease because of increasing age, your participation will help us to learn about these new risk factors in a group of people who are at very low risk for developing cardiovascular disease. You will also help us to learn what happens to the new risk factors after 10 days of no physical activity. You might benefit because you will receive information about your current cardiovascular fitness and body fat levels, and you will be told of any abnormalities found during testing and will be advised to consult your personal physician. You will also be told of your results of the different risk factors that we measure and will be provided an explanation of these results.

Emergency Information: In the event of a physical injury resulting from participation in this study, medical attention is available at the Washington Adventist Hospital. The University of Maryland does not provide any medical, hospitalization, or other insurance for participants in this research study nor will the University of Maryland provide any medical treatment or compensation for any injury sustained as a result of participation in this research study except as required by law.

Contact Information: This research is being conducted by James Hagberg PhD of the Department of Kinesiology at the University of Maryland. If you have any questions about the research study itself, please contact Dr. Hagberg at 301-405-2487 (office) or via email at hagberg@umd.edu. If you have questions about your rights as a research subject or wish to

report a research-related injury, please contact: **Institutional Review Board Office, University of Maryland, College Park, MD 20742; email: irb@deans.umd.edu; telephone: 301-405-0678**.

This research has been reviewed according to the University of Maryland College Park IRB procedures for research involving human subjects.

Your signature below indicates that you are at least 18 years of age, the research has been explained to you, your questions have been fully answered, and you freely and voluntarily choose to participate in this research program.

____
Participant's printed name

________________________________ ______________________
Participant's signature Date

________________________________ ______________________
Witness Signature Date

________________________________ ______________________
Investigator Signature Date

Project Title: **Life-Long Exercise and Novel Cardiovascular Disease and Aging Risk Factors – Young Sedentary Participants**

CONSENT FORM

This is a research project being conducted by Dr. James Hagberg in the Department of Kinesiology at the University of Maryland College Park. We are inviting you to participate in this research project because you are 18 – 35 years of age, are generally healthy, and are generally sedentary. The purpose of this study is to determine whether aging and regular exercise are associated with novel risk factors related to cardiovascular disease. In this case "novel" risk factors mean newer risk factors beyond the standard risk factors of cholesterol levels, blood pressure, diabetes, and obesity. Two groups of older participants, one consisting of life-long exercisers and the other of generally sedentary individuals, have already completed the study. We recruit you for the study because we would like to compare the levels of novel risk factors from these older groups to those from younger individuals who do not exercise regularly. Your participation in this research is completely voluntary and you may choose not to take part at all. You are free to ask questions at any time without penalty. If you decide to participate in this research, you may stop participating at any time. If you decide not to participate in this project or if you stop participating at any time, you will not be penalized or lose any benefits to which you otherwise qualify. The specific tests, their requirements, and time commitments are described below.

Participant Characteristics: You have already completed a telephone or personal interview that you verbally consented to that determined that you are 18 – 35 years of age, are not a diabetic, have no evidence of cardiovascular or lung disease, and have no other medical problems that keep you from exercising vigorously. We have also discussed with you that certain medications may exclude you from taking part in this study.

Study Procedures: If you qualify and complete this study, your total involvement will consist of 2 visits over approximately 1 week, including 1 Screening Visit and 1 Testing Visit.

Screening Visit: For the Screening Visit, the study will be explained to you, your medical history will be reviewed, and you will be asked to provide your written informed consent. Your resting blood pressure will be measured and about 2 tablespoons of blood will be drawn from a vein in your arm. Your body composition will be assessed by a procedure that involves measuring the thickness of your skinfolds. You will then undergo a treadmill exercise test to determine your exercise capacity. This test will be done on an exercise treadmill where the treadmill incline will increase every 2 minutes until you cannot continue. Blood pressure, heart rate, and the electrical activity of your heart (electrocardiogram) will be recorded before, during, and after the test. During this test you will have a noseclip on your nose and you will breathe through a mouthpiece so that the air that you breathe out can be analyzed. This visit will last about 1 hour.

Testing Visit: For the Testing Visit, you will report to the laboratory in the morning after an overnight fast (only taking in water from 8 PM the night before). Prior to this visit you will record all items that you eat over a 7 day period. About 4 tablespoons of blood will be drawn from a vein in your arm. You will then undergo treadmill exercise for 30 minutes at a moderate to vigorous intensity. Heart rate and blood pressure will be monitored before, during, and after

the exercise. During this test you will have a noseclip on your nose and you will breathe through a mouthpiece so that the air that you breathe out can be analyzed. At 5 and 30 minutes after the exercise approximately 4 tablespoons of blood will be drawn from a vein in your arm. The blood samples that you provide before and after exercise will be tested for levels of the new cardiovascular disease risk factors. This visit will last about 1.5 hours.

The maximum total amount of blood that will be drawn during this Testing is about 12 tablespoons. This is approximately 1/3 of the amount of blood that is typically drawn during a single blood donation. These samples will be used to measure a number of chemicals and cell numbers in the blood that may be related to a person's risk of cardiovascular disease.

☐ Yes, I consent to having my blood drawn and saved for future analyses
☐ No, I do not consent to having my blood drawn and saved for future analyses and my samples must be destroyed after the completion of this study.

Confidentiality: We will do our best to keep your personal information confidential. To help protect your confidentiality, all data are kept in a locked office with access available only to study personnel. Furthermore, all computer data bases containing results from this study will not have any names attached to them. If we write a report or article about this research project, your identity will be protected to the maximum extent possible. Your information may be shared with representatives of the University of Maryland College Park or government authorities if you or someone else is in danger or if we are required to do so by law.

Samples of your blood will be sent to other collaborating laboratories for other blood measurements. Your blood samples sent to these laboratories will be identified only by a numeric code and only investigators at the University of Maryland College Park will know whose name is associated with each coded number. The list of names and codes will be retained at the University of Maryland College Park for up to 25 years.

Risks: The following risks are associated with your participation in this study. (1) There is some risk associated with maximal exercise testing but complications are unusual in healthy individuals in your age group (Gibbons et al., 1989, *Circulation* Vol. 80, No. 4). Risks will be minimized by having the test administered by personnel trained in such tests and emergency procedures. You will gradually cool down upon reaching your highest effort, which will further help to reduce the risk of complications. A cardiac defibrillator will be present at all testing sessions. (2) There are risks of bruising and infection associated with blood drawing. These risks will be minimized by using sterile techniques and by having experienced personnel draw all blood samples. (3) The skinfold body composition testing has no known risks. (4) There are no known risks associated completing 7 day dietary records. (5) Your involvement will require some amount of time, travel, and effort. Your parking costs will be covered.

Benefits: This study may not help you personally, but may help the investigators to determine whether regular exercise results in better levels of new cardiovascular disease risk factors. Because you are a young and healthy participant, and you are therefore not at elevated

risk for cardiovascular disease because of increasing age, your participation will help us to learn about these new risk factors in a group of people who are at very low risk for developing cardiovascular disease. You might benefit because you will receive information about your current cardiovascular fitness and body fat levels, and you will be told of any abnormalities found during testing and will be advised to consult your personal physician. You will also be told of your results of the different risk factors that we measure and will be provided an explanation of these results.

Emergency Information: In the event of a physical injury resulting from participation in this study, medical attention is available at the Washington Adventist Hospital. The University of Maryland does not provide any medical, hospitalization, or other insurance for participants in this research study nor will the University of Maryland provide any medical treatment or compensation for any injury sustained as a result of participation in this research study except as required by law.

Contact Information: This research is being conducted by James Hagberg PhD of the Department of Kinesiology at the University of Maryland. If you have any questions about the research study itself, please contact Dr. Hagberg at 301-405-2487 (office) or via email at hagberg@umd.edu. If you have questions about your rights as a research participant or wish to report a research-related injury, please contact: **Institutional Review Board Office, University of Maryland, College Park, MD 20742; email: irb@deans.umd.edu; telephone: 301-405-0678**.

Project Title: **<u>Life-Long Exercise and Novel Cardiovascular Disease and Aging Risk Factors – Young Sedentary Participants</u>**

This research has been reviewed according to the University of Maryland College Park IRB procedures for research involving human participants.

Your signature below indicates that you are at least 18 years of age, the research has been explained to you, your questions have been fully answered, and you freely and voluntarily choose to participate in this research program.

____
Participant's printed name

___________________________________   _______________________
Participant's signature   Date

___________________________________   _______________________
Witness Signature   Date

___________________________________   _______________________
Investigator Signature   Date

CONSENT FORM

Project Title	**Effect of endurance exercise on angiogenic cells and growth factors during postprandial lipemia.**
Why is this research being done?	This is a research project being conducted by James M. Hagberg, Ph.D. at the University of Maryland, College Park. We are inviting you to participate in this research project because you are a healthy, physically active male between the ages of 18 and 35 with no history of cardiovascular or metabolic disease. You have already indicated by phone or email interview that you meet these criteria and also that you have no history of lactose intolerance (an inability to digest dairy products) or other disorders of the digestive system. The purpose of this research project is to investigate the effects of a high-fat meal on recently-discovered proteins and cell types in the blood. We are also interested in whether exercise performed on the day before eating a high fat meal alters these effects. Because most of us in modern Western societies are almost always in a "postprandial" state (i.e., we have usually had a recent large meal), the results of this study will have important implications for a large number of people. The experimental protocol (described in detail below) involves performing exercise or no exercise on the day before a "postprandial lipemia" test. Postprandial lipemia simply means measuring the levels of fat (triglycerides) appearing in your blood after eating a high-fat meal (a chocolate milkshake).
What will I be asked to do?	This study involves 5 visits to the laboratory, totaling 15.5 hours of your time: Visit #1: ASSESSMENT OF BODY COMPOSITION, CARDIOVASCULAR RISK FACTORS, AND AEROBIC CAPACITY. On the 1st visit, you will read and sign the informed consent, and complete and submit the physical activity history and medical history forms. Your height will then be measured on a metric scale and body composition will be assessed using the skinfold method, in which skinfold thickness is measured using calipers at 7 sites on your body. Blood pressure will be measured, and a fasting blood sample will be obtained (1 tsp). The purpose of this blood draw is to exclude any subjects from further testing who have blood cholesterol levels: LDL or "bad" cholesterol above 130 mg/dl; HDL or "good" cholesterol below 40; or total cholesterol above 200 mg/dl). Subjects with high blood glucose

| | values (i.e., above 100 mg/dl) will also be excluded from further testing. Following the body composition assessment and blood draw, your maximal oxygen uptake (a measure of the fitness of your cardiovascular system) will be tested on a stationary bicycle. This test will begin at a low intensity, and the intensity will increase every two minutes until you can no longer continue. The test is designed to last ~6-12 minutes. Your expired air will be collected using a specialized mouthpiece and analyzed. This visit will last a total of 1 to 1.5 hours.

Visit #2: CONTROL TRIAL OR EXERCISE TRIAL. For the 2nd and 4th visits, you will report to the laboratory at 4:00 PM having not ingested any food 3 hours before the treatment protocol. The order of the control and exercise treatments will be randomized. This visit will last approximately 1.5 hours.

EXERCISE TRIAL: You will be allowed to ingest only water during the exercise treatment and the ingested volume will be recorded. The exercise protocol will consist of cycling at an intensity corresponding to 70% of your maximal exercise capacity measured during visit #1. You will exercise until you have burned 600 Calories. We anticipate that you will perform about 45 minutes of exercise (between 30 and 60 minutes) to burn 600 Calories at this exercise intensity. Furthermore, after your maximal exercise test (visit #1), we will be able to calculate an estimate of the number of minutes it will take you to burn 600 calories. However, the equation we use is accurate for most individuals, but it is not perfect for everyone. Therefore, we will not know exactly how long you will need to exercise on this visit until you actually perform the experiment. The estimate we give you has a margin of error of approximately 5 minutes for most people.

CONTROL TRIAL: You will perform the no-exercise control trial by reporting to the laboratory at 5 PM and sitting quietly for the same length of time as is estimated for the exercise trial.

Following both the exercise and control trials, you will be given the evening postexercise meal to take home and eat between 7:00 p.m. and 7:30 p.m. This meal will contain two Zone Perfect® Nutrition Bars. The meal will provide 420 kcals and will contain no more than 40% carbohydrate (42 g), 30 % fat (14 g), and 30 % protein (32 g). You will be asked not to eat anything more after |

	the evening postexercise meal until your next visit at ~7:00 a.m. on the following day, and to drink only water during this time. You may drink as much water you like and to record the volume on the dietary log. Visit #3: POSTPRANDIAL LIPEMIA TEST #1. You will report to the laboratory between 7:00 and 7:15 A.M. in a fasted state as soon as possible after waking up, having performed no physical activity during that morning. You will have an intravenous (i.e., "IV") catheter inserted into an easily accessible vein on your arm by ~7:30 a.m. You will consume the test meal from ~7:30 A.M. until ~08:00 A.M. The amount you will consume will be determined on the basis of your body size. An average serving of this test meal provides 1362 calories (84% fat). The ingredients are heavy whipping cream, chocolate syrup, sugar, and milk powder. It is very much like a rich, chocolate milk shake. Immediately upon completion of the meal, a blood sample will be obtained for the 0-hr time point. The next blood samples will be obtained at 60 minute intervals after meal ingestion for 4 hours. The venous catheter will be flushed a standard saline solution after each sample is obtained to prevent blood from clotting in the line. You will be allowed to drink water throughout the test and the volume you drink will be recorded. The following volumes of blood will be obtained at each sampling point. The purpose of each sample is including in parentheses. <u>Baseline (pre-meal):</u> 4 tsp for examination of fasting triglycerides and angiogenic growth factor levels <u>0 hr (immediately upon completion of meal):</u> 8 tsp for isolation of stem cells measuring angiogenic growth factors <u>1, 2, and 3 hrs:</u> 4 tsp for measuring angiogenic growth factors and triglyceride concentrations <u>4 hr:</u> 8 tsp for isolation of stem cells and measuring angiogenic growth factors Therefore, a total of 32 tsp of blood will be obtained during this test. For comparison, a pint (96 tsp) is given at a blood donation. This test will last approximately 5.5 hours. Visit #4: EXERCISE OR CONTROL TRIAL (as described for

	Visit #2). The remaining experimental trial will be completed on this visit. This visit will occur at least 3 and not more than 10 days after the preceding postprandial lipemia test. This visit will last approximately 1.5 hours. Visit #5: POSTPRANDIAL LIPEMIA TEST #2 (exactly as described for Visit #3). This visit will last approximately 5.5 hours.

What about confidentiality?	We will do our best to keep your personal information confidential. To help protect your confidentiality, you will be identified by a 2-digit ID number on data forms, questionnaires, and computer files. Your name will not be included on any of these forms. Confidentiality will be maintained by developing a code form that associates the ID numbers with the names of the participants. The code form will be maintained in a locked, secure filing cabinet in the Metabolic Testing Laboratory, and only study personnel will have access to this file. All study forms/questionnaires will include subjects' ID numbers only (no names). If we write a report or article about this research project, your identity will be protected to the maximum extent possible. Your information may be shared with representatives of the University of Maryland, College Park or governmental authorities if they or others are in danger or if we are required to do so by law. Data will be kept for a minimum of 3 years and a maximum of 10 years. Information collected from excluded participants or participants who withdraw from the study will not be used and will be destroyed or shredded immediately. Responses from participants recruited by email will be kept confidential by printing paper copies of emails and storing them in locked, secured files according to subject codes. All personally identifying information (e.g., name and email address) will be redacted from the printed versions and, all email correspondence containing any identifying information will be permanently deleted after securing the hard printed copies.

What are the risks of this research?	Participation in this study does not involve any known psychological, social, legal, or economic discomfort or risks. Physical discomfort may be experienced during exercise along with minor local discomfort due to the insertion of a venous

	catheter. The primary risks in this study are (1) physical and mental fatigue due to the performance of maximal and submaximal exercise, and (2) physical discomfort from blood drawing, The risks of exercise will be minimized by: (1) recruiting subjects such as yourself who are accustomed to vigorous exercise and who have no history of cardiovascular or metabolic disease, (2) informing you about the symptoms and signs of strenuous exercise complications and instructing you to stop exercising if these symptoms or signs develop, (3) monitoring behavior continuously and terminating exercise immediately if there are signs of developing illness such as headache, nausea, mental disorientation, lack of coordination, or dizziness. Should you develop any signs or symptoms, an emergency protocol will be immediately initiated and emergency medical technicians will be called. The probability of avoiding serious injury is high if emergency care is initiated immediately. Emergency protocols for handling a cardiovascular problem are posted in the laboratory at all times and the study personnel are trained and certified to perform emergency CPR/AED procedures. All exercise testing procedures and safety protocols in our laboratory follow the American College of Sports Medicine's guidelines for exercise testing. The risks of drawing blood from the arm include the possibilities of feeling lightheaded or fainting, development of a small bruise, or infection. These risks will be minimized by using only qualified and experienced personnel to draw blood who will follow standard sterile techniques, who will observe the subject after the catheter is withdrawn, and who will apply pressure to the blood-draw site. Additionally, there may be some risk associated with consuming a high fat meal, including indigestion and/or nausea. However, it is anticipated that this risk will not be substantially greater than that faced in day-to-day life, as the test meal is very much like a rich, chocolate milkshake that might be purchased from a common fast food chain. The risk of gastrointestinal distress is minimized by ensuring that you have no history of lactose intolerance or gastrointestinal disorders. All testing will be carefully supervised by the investigators and other trained personnel to reduce the possible risks. In the unlikely event of an emergency, laboratory personnel trained in CPR will be present during all test sessions. An AED is present in the laboratory at all times and is verified to be in proper

	working order on a daily basis.
What are the benefits of this research?	This study may not help you personally. However, you might benefit by having their body composition assessed and will be provided information about your height, weight, body fat levels, aerobic fitness, your current levels of cardiovasuclar disease risk factors. Finally, you will be helping us understand the effects of exercise and postprandial lipemia on a recently-discovered population of cells that repair the cardiovascular system. Therefore, you will be contributing to the advancement of important biomedical research by volunteering your time.
Do I have to be in this research? May I stop participating at any time?	**Your participation in this research is completely voluntary. You may choose not to take part at all. If you decide to participate in this research, you may stop participating at any time. If you decide not to participate in this study or if you stop participating at any time, you will not be penalized or lose any benefits to which you otherwise qualify.**
Is any medical treatment available if I am injured?	The University of Maryland does not provide any medical, hospitalization or other insurance for participants in this research study, nor will the University of Maryland provide any medical treatment or compensation for any injury sustained as a result of participation in this research study, except as required by law.
What if I have questions?	This research is being conducted by Dr. James M. Hagberg at the University of Maryland, College Park. If you have any questions about the research study itself, please contact Dr. Hagberg at (301) 405-2487 or by email at hagberg@umd.edu. If you have questions about your rights as a research subject or wish to report a research-related injury, please contact: **Institutional Review Board Office, University of Maryland, College Park, Maryland, 20742; (e-mail) irb@umd.edu; (telephone) 301-405-0678** This research has been reviewed according to the University of Maryland, College Park IRB procedures for research involving human subjects.
Statement of Age of Subject and Consent	**Your signature indicates that:** **you are at least 18 years of age;,** **the research has been explained to you;** **your questions have been fully answered; and** **you freely and voluntarily choose to participate in this research project.**
Subject Signature	**NAME OF SUBJECT** |

and Date		
	SIGNATURE	
	DATE	
Witness Signature and Date	**NAME OF WITNESS**	
	SIGNATURE	
	DATE	
Investigator Signature And Date	**NAME OF INVESTIGATOR**	
	SIGNATURE	
	DATE	

Appendix C – Permission from the American Physiological Society to include Published Material in Dissertation (Studies 1 and 2).

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Publisher (if journal or book): n/a

URL (if website): n/a

Date of Meeting or Publication: July 7, 2011

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APPROVED
By pripka at 11:42 am, May 03, 2011

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Affiliation: University of Maryland College Park

University Address (for PhD students): 0224 SPH Building, 255 Valley Dr

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Year: ______ DOI: 10.1152/japplphysiol.00541.2011

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Dissertation Title: "Regulatory Effects of Acute and Chronic Endurance Exercise on Nitric Oxide and Reactive Oxygen Species in Human Circulating Angiogenic Cells". This article will be included as Ch. 4.

Publisher (if journal or book): ______

URL (if website): ______

Date of Meeting or Publication: August 2012

Will readers be charged for the material: ○ Yes ○ No

Additional Information:

The article will be included in my dissertation (defense date is July 7, 2011). Dissertation will be submitted to the UMD graduate school in August 2011, and will be published on the UMD Libraries web site in PDF form after 1 year (August 2012).

APPROVED
By pripka at 8:22 am, Jun 28, 2011

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Rita Scheman
Publications Manager & Executive Editor

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