MODULATION OF VASCULAR ENDOTHELIAL FUNCTION BY CHRONIC LOW-GRADE INFLAMMATION IN HEALTHY MIDDLE-AGED AND OLDER ADULTS: INFLUENCE OF HABITUAL AEROBIC EXERCISE

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Modulation of vascular endothelial function by chronic low-grade inflammation in healthy middle-aged and older adults: influence of habitual aerobic exercise

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Impaired vascular endothelium-dependent dilation (EDD), a bio-marker of vascular endothelial dysfunction, is an independent predictor of future cardiovascular events. EDD is reduced with age, but is preserved in middle-aged and older (MA/O) adults who habitually perform aerobic exercise. The purpose of these studies was to determine the role of chronic low-grade inflammation, as assessed by white blood cell (WBC) count and endothelial nuclear factor-κB (NF-κB) signaling, in the modulation of EDD in sedentary and aerobic exercise-trained MA/O adults.

EDD was 34% less in MA/O sedentary adults with higher compared with lower WBC count. Vascular smooth muscle responsiveness to nitric oxide (NO) was 18% less in subjects with higher vs. lower WBC count, but did not fully explain the differences with EDD. Inhibition of NO reduced EDD in subjects with lower, but not higher WBC count. Tetrahydrobiopterin selectively improved EDD in subjects with higher WBC count by increasing NO bioavailability. Thus, among healthy sedentary MA/O adults, a higher WBC count is related to impaired EDD and this is mediated by reduced responsiveness to NO and reductions in tetrahydrobiopterin and NO bioavailability.

MA/O sedentary adults had a greater expression of NF-κB, a key pro-inflammatory transcription factor, in their vascular endothelial cells compared with young sedentary and MA/O aerobic exercise-trained adults. In a sub-group of MA/O subjects, oral salsalate was used to inhibit NF-κB signaling. Salsalate treatment reduced endothelial NF-κB expression in sedentary subjects, but had no effect in exercise-trained subjects. EDD improved by 76% with salsalate in the sedentary adults, whereas there was no improvement in the exercise-trained adults. In

sedentary subjects, antioxidant vitamin C infusion improved EDD by 32% during the placebo condition, but had no effect during salsalate. In exercise-trained subjects, vitamin C infusion did not change EDD during either treatment. Therefore, in sedentary MA/O adults, increased NF-κB signaling suppresses EDD, in part, by increased oxidative stress. Reduced NF-κB signaling is a key mechanism for preserved EDD in exercise-trained MA/O adults.

In conclusion, chronic low-grade inflammation impairs EDD in healthy sedentary MA/O adults. However, MA/O adults who habitually perform aerobic exercise have preserved EDD as a result of reduced inflammatory signaling.

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CHAPTER I

List of Publications

Research Articles

Walker AE, Kaplon RE, Seibert SM, Seals DR. Fenofibrate Improves Vascular Endothelium-Dependent Dilation in Healthy Middle-Aged and Older Adults. In preparation.

Walker AE, Pierce GL, Kaplon RE, Lesniewski LA, Donato AJ, Seals DR. Absence of Nuclear Factor-κB-Mediated Suppression of Vascular Endothelial Function in Middle-Aged and Older Adults Who Habitually Perform Aerobic Exercise. To be submitted.

Jablonski KL, Chonchol M, Pierce GL, **Walker AE**, Seals DR. 25-Hydroxyvitamin D Deficiency is Associated with Vascular Endothelial Dysfunction in Middle-Aged and Older Adults. In Press *Hypertension.*

Pierce GL, Jablonski KL, **Walker AE**, Seibert SM, Black SM, Sharma S, Seals DR. Reduced Tetrahydrobiopterin Bioactivity Contributes to Decreased Carotid Artery Compliance with Aging in Healthy Adults. Submitted to *J Hypertens.*

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CHAPTER II

Introduction

The vascular endothelium plays a critical role in the development of atherosclerotic diseases, such as coronary artery disease, that are the main contributors to cardiovascular-related illness and premature death. Impaired vascular endothelium-dependent dilation (EDD) is the central feature of vascular endothelial dysfunction and is an independent predictor of future clinical atherosclerotic disease and related events. Middle-aged and older adults are at a greater risk for cardiovascular diseases and have an impaired EDD compared with young adults. Thus, it is biomedically important to identify factors that modulate EDD among middle-aged and older adults. Chronic low-grade inflammation and elevated inflammatory signaling are possible modulators of EDD.

A greater white blood cell (WBC) count within the clinically normal range is predictive of future cardiovascular events and related to impaired EDD among patients with clinical disease. However, it is unknown if WBC count relates to EDD in healthy middle-aged and older adults. In Chapter IV, I tested the hypothesis that WBC count is related to EDD in healthy middle-aged and older adults and this is mediated by reduced tetrahydrobiopterin and nitric oxide bioavailability in adults with higher WBC count. This study was able to establish a relation between systemic low-grade inflammation and EDD. However, the study design could only identify an association and could not determine cause-and-effect. Likewise, the study examined a systemic marker of inflammation, but could not establish local inflammatory signaling in the endothelial cell.

To investigate the role of local inflammatory signaling, a key pro-inflammatory transcription factor, nuclear factor-κB (NF-κB), was studied. Endothelial cells protein expression of NF-κB is increased in the whole cell and nucleus (a marker of activity) with aging in humans.

NF-κB activity can be inhibited with oral salsalate administration in humans and, thus, cause-and-effect of inflammatory signaling can be established. In Chapter V, I hypothesized that NF-κB activity impairs EDD in healthy middle-aged and older adults.

Middle-aged and older adults who habitually perform aerobic exercise have a lower risk of cardiovascular events and an enhanced EDD compared with their non-exercising peers. However, the mechanism for these effects of aerobic exercise are unknown, particularly related to the role of inflammatory signaling. In Chapter V, I also tested the hypothesis that aerobic exercise-trained middle-aged and older adults have an absence of NF-κB -mediated suppression of EDD.

If inflammatory signaling has a role in the impaired EDD in sedentary and preserved EDD in aerobic exercise-trained middle-aged and older adults, then it would be important to identify related mechanisms. Oxidative stress and inflammatory signaling act synergistically to amplify and promote the actions of each other. Thus, in Chapter V, I hypothesized that NF-κB-mediated suppression of EDD is a result of increased oxidative stress in sedentary middle-aged and older adults and that oxidative stress-mediated suppression of EDD is absent in aerobic exercise-trained middle-aged and older adults.

Specific Aims

Specific Aim 1 (Chapter IV): To determine if WBC count is related to EDD in sedentary healthy middle-aged and older adults.

Specific Aim 2 (Chapter IV): To determine if reduced tetrahydrobiopterin and nitric oxide bioavailability are responsible for the lower EDD in sedentary healthy middle-aged and older adults with higher compared with lower WBC count.

Specific Aim 3 (Chapter V): To determine if NF-κB activity suppresses EDD in healthy sedentary middle-aged and older adults. In addition, to determine if NF-κB-mediated suppression of EDD is absent in healthy aerobic exercise-trained middle-aged and older adults.

Specific Aim 4 (Chapter V): To determine if oxidative stress has a role in the presence of NF-κB-mediated suppression of EDD in sedentary healthy middle-aged and older adults. Likewise, to determine if oxidative stress has role in the absence of NF-κB-mediated suppression of EDD in aerobic exercise-trained healthy middle-aged and older adults.

CHAPTER III

Review of Literature

Clinical Significance

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in the United States and other industrialized societies. Atherosclerotic diseases, such as coronary artery disease, are the main contributors to CVD-related illness and premature death (51). It is now appreciated that the vascular endothelium is the critical site for development of atherosclerosis (8, 81).

The endothelium is a single layer of cells lining the lumen of the vessel wall that interfaces with the blood. The location of the endothelium is important to its function as it allows the cells to sense hemodynamic changes and interact with circulating substances. The endothelium is an autocrine and paracrine organ that releases factors to regulate vasodilation, vasoconstriction, coagulation and cell adhesion. Vascular endothelial dysfunction is defined as a general alteration in endothelial cell function and exhibits an atherogenic phenotype characterized by pro-vasoconstriction, pro-coagulation, pro-inflammatory and pro-vascular smooth muscle proliferation properties (77, 81). Impaired vascular endothelium-dependent dilation (EDD) is the central feature of vascular endothelial dysfunction and is an independent predictor of future clinical atherosclerotic disease and related events (42, 69, 84, 85).

EDD

Impaired EDD results primarily from reduced bioavailability of nitric oxide (NO) mediated by reduced endothelial NO production and/or enhanced conversion of NO to other products by reactive oxygen species (ROS) (14). NO, a free radical gas with a short half-life, is the principal vasodilator released by the endothelial cell (8). Because NO is small and electrically neutral it

can rapidly diffuse across membranes without the need for a channel or transporter (15) and, therefore, can diffuse to adjacent vascular smooth muscle cells to trigger relaxation. In the smooth muscle cells, NO increases the activity of guanylate cyclase and, thus, increases the rate for conversion of GTP to cGMP (79). cGMP decreases intracellular calcium causing the smooth muscle to relax (2).

NO is produced during the oxidation of L-arginine to L-citrulline in the reaction catalyzed by NO synthase (NOS). In the vascular endothelium, endothelial NOS (eNOS) is a constitutively expressed enzyme (59). In the resting state, eNOS is bound to caveolin-1 in caveolae (invaginations of the cell membrane). Endothelial stimuli such as acetylcholine, bradykinin, and shear stress increase intracellular calcium concentrations and calcium (by interaction with calmodulin) displaces caveolin-1, thus releasing eNOS to the cytosol where it can be catalytically active (3, 66). eNOS activity can also increase in a calcium-independent manner by phosphorylation at Ser^{1179} (3).

Tetrahydrobiopterin (BH4) is an important eNOS cofactor. The exact role of BH4 in eNOS function is uncertain, but possibilities include stabilizing the eNOS dimer, promoting the coupling of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation to NO synthesis, facilitating the binding to L-arginine, protecting the enzyme from inactivation, and functioning as a redox active cofactor (3). In the presence of high BH4 cellular concentrations, eNOS will produce only NO. However, when concentrations of BH4 are low, both NO and superoxide can be produced by eNOS (52). In the low BH4 environment, this occurrence is known as “eNOS uncoupling” because the oxidation of NADPH is no longer coupled to the formation of NO (3).

Measurement of EDD in Humans. Measurement of EDD in the coronary arteries is considered the gold standard of endothelial assessment, but this technique is invasive, risky and expensive (77). However, endothelial dysfunction is systemic, and EDD in peripheral arteries: a) correlates with EDD in the coronary circulation (77); and b) predicts future cardiovascular events in patients with heart disease (42, 69, 84, 85). There are two common

methods to assess EDD in peripheral arteries: forearm blood flow (FBF) response to endothelium-dependent dilators and flow-mediated dilation (FMD).

The FBF model measures EDD by the vasodilation response to an endothelium-dependent agonist (e.g., acetylcholine). This method utilizes pharmacologic infusions into the brachial artery and the resulting FBF response is measured with strain-gauge venous occlusion plethysmography. An increase in FBF reflects the dilation of the resistance vessels of the forearm to the respective agonist (47, 82). The advantage of this procedure is it allows for "pharmaco-dissection" of mechanisms involved in EDD. For example, the role of NO can be assessed by inhibition of NOS with N^G-monomethyl-L-arginine (L-NMMA) and the role of BH4 can be assessed by infusion of this compound. A dose-response curve is established for each condition and differences are identified in either the slopes of these curves or the peak blood flows attained.

The FMD technique is non-invasive and utilizes B-mode ultrasound to measure the diameter of a peripheral conduit artery (most commonly the brachial). Dilation is stimulated by an increase in shear stress on the vessel wall. A small blood pressure cuff, typically positioned around the forearm, is inflated to supra-systolic pressure for several minutes to cause ischemia in the tissue distal to the cuff. When the pressure in the cuff is rapidly released, the hyperemic stimulus increases the shear stress on the endothelial surface. The diameter of the artery is measured for two minutes after the cuff release (18, 40). An advantage to the FMD technique is the stimulus for dilation is physiological (increased shear stress)(52). The dilation in response to increased shear stress is primarily a result of increased release of NO from the endothelial cell, but other factors such as prostanoids may also mediate shear induced dilation (18). Unlike the FBF technique that assesses EDD of resistance vessels, FMD assesses EDD of conduit arteries.

If a treatment increases or decreases EDD, then it is also important to know if endothelium-independent dilation has changed. Exogenous NO donors are used to assess

endothelium-independent dilation, commonly sodium nitroprusside for the FBF model and sublingual administration of nitroglycerin for the ultrasound technique (18, 82). This response is intended to show if vascular smooth muscle relaxation to NO differs between groups or conditions and, therefore, might contribute to differences in EDD observed.

Inflammatory signaling, oxidative stress and EDD

Atherosclerosis is now recognized as a chronic inflammatory disease of the vasculature (68). Inflammatory processes are involved in all stages of atherogenesis from early endothelial activation by oxidized/modified lipids to rupture of the atherosclerotic plaque (39). Increased vascular inflammatory signaling is commonly associated with elevated circulating (systemic) concentrations of pro-inflammatory cytokines and acute-phase proteins and reduced anti-inflammatory cytokines (7, 55).

Several observations indicate that vascular endothelial dysfunction in humans is related to inflammatory signaling:

1) circulating concentrations of pro-inflammatory cytokines are inversely, whereas anti-inflammatory cytokines are positively, related to EDD (32, 72, 78);

2) patients with clinical atherosclerotic diseases as well as individuals with risk factors for CVD have increased plasma concentrations of pro-inflammatory cytokines and decreased EDD (10, 33);

3) intravenous infusion of pro-inflammatory cytokines impairs EDD in healthy adults (44, 61, 67);

4) administration of a vaccine that causes a mild temporary inflammatory response reduces EDD in healthy adults, an effect that is related to increases in plasma concentrations of pro-inflammatory cytokines (44) and is prevented by pre-treatment with aspirin (48).

The exact mechanisms by which inflammatory signaling impairs EDD in humans are incompletely understood. One hypothesis is that inflammatory signaling suppresses EDD by increasing oxidative stress, a state in which the production of ROS is increased relative to antioxidant defenses. Inflammatory signaling stimulates oxidant enzyme systems (e.g., NADPH oxidase) to produce ROS, including superoxide anion (75). Superoxide, produced in the vessel wall or by immune cells, reacts rapidly with NO to form peroxynitrite, thus reducing NO bioavailability. A temporary increase in inflammatory-stimulated ROS production can activate antioxidant enzyme expression to maintain cell redox balance. However, persistent elevations in ROS formation (e.g., with chronic low-grade inflammatory signaling) resets the redox state such that antioxidant production is not effectively stimulated, allowing the establishment of oxidative stress (26). In addition to inflammatory signaling promoted oxidative stress, increased ROS also stimulates pro-inflammatory gene transcription and protein expression via the redox-sensitive pro-inflammatory transcription factor nuclear factor κ B (NF-κB) (17, 46). Thus, oxidative stress and inflammatory signaling act synergistically to amplify and promote the actions of the other.

The direct and indirect actions of pro-inflammatory cytokines on EDD are produced via intracellular signaling pathways, several of which activate NF-κB. NF-κB is an important nuclear transcription factor that consists of several isoforms, the most abundant of which is the p65/p50 complex. NF-κB is believed to regulate >160 genes involved in determining cellular inflammatory and redox states, including those genes encoding for cytokines, chemokines and leukocyte adhesion molecules (21). Some of these molecules, in turn, stimulate the release of acute phase proteins from hepatocytes (7). NF-κB is normally confined to the cytoplasm by inhibitor of NF-κB (I-κB). The phosphorylation of I-κB by I-κB kinase (IKK) releases NF-κB and allows it to translocate into the nucleus. IKK is activated by pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), intracellular ROS and oxidant-modified lipoproteins (21). It is important to emphasize that NF-κB activation is not atherogenic *per se* and is necessary to

mount an appropriate acute inflammatory response (21). However, chronic activation of NF-κB can result in a pro-inflammatory/pro-oxidant endothelial phenotype that leads to impaired EDD (21, 37). Indeed, reduced endothelial NF-κB activation in humans by treatment with salsalate, a non-acetylated salicylate, improved EDD in overweight and obese adults (65).

The number of circulating immune cells, indicated by white blood cell (WBC) count, can be a marker of an immune response. Within the normal clinical range, higher WBC count is associated with increased risk of future cardiovascular events (36, 57). WBC count is inversely related EDD in smokers and among patients with clinical diseases, such as type 2 diabetes mellitus and hypertension (29, 53, 83). The influence of WBCs on EDD could be mediated by local interactions with the vascular wall as WBCs are constantly in contact with the endothelium via rolling, adhesion and infiltration into the vascular wall (63, 68). On interacting with the vascular wall, WBCs can produce and release ROS and cytokines, which could, in turn, influence gene and protein expression, intracellular signaling and vasodilatory responsiveness (16). Myeloperoxidase, produced by populations of WBCs, is a pro-oxidant enzyme and a predictor of EDD in patients with clinical disease (56, 76). Myeloperoxidase leads to reduced NO bioavailability by two major mechanisms: a) myeloperoxidase catalyzes the reaction of hydrogen peroxide and NO to produce nitrogen dioxide, thus consuming NO; and b) myeloperoxidase produces ROS that can oxidize BH4 and lead to eNOS uncoupling (1, 28).

Reduced EDD with sedentary aging

Aging is a major risk factor for clinical atherosclerotic diseases. Recent findings from the Framingham Study show that age is a strong independent correlate of EDD (58). Marked impairments in EDD (with intact endothelium-independent dilation) are observed in older adults even in the absence of clinical disease or significant risk factors for CVD (23, 31, 35). Thus, adult aging results in vascular endothelial dysfunction as indicated by impaired EDD. Unfortunately, until recently vascular aging was viewed as an unavoidable consequence of the

aging process and, therefore, little information is available on possible preventive strategies and underlying mechanisms.

Reduced EDD with age is a result of reduced NO bioavailability. Arteries of older rats have less NO bioavailability compared with arteries from young rats (38). Moreover, the reduction in FBF in response to intra-brachial artery infusion of L-NMMA is smaller in older compared with young adult humans (70) indicating lower NO-stimulated EDD in older age. Possible mechanisms that contribute to reduced NO bioavailability with age are increased inflammatory signaling and oxidative stress.

Aging, inflammatory signaling and oxidative stress. Aging is considered a state of chronic low-grade inflammatory signaling characterized by increased circulating concentrations of pro-inflammatory cytokines and acute phase proteins (4, 12, 50), with accompanying vascular pro-inflammatory signaling (73). Even in the absence of CVD risk factors or clinical atherosclerotic disease, older adults have a 2-4 fold increase in plasma concentrations of cytokines TNF-α (11, 13, 62), interleukin (IL)-6 (30, 80), and acute phase protein C-reactive protein (4). Analysis of endothelial cells obtained from veins of young and older adult humans indicates increased IL-6 and TNF-α protein expression with age (24). In humans, impaired brachial artery FMD with aging is related to low-grade pro-inflammation as indicated by circulating inflammatory markers (78). Thus, it appears that both systemic and vascular pro-inflammatory signaling is elevated with aging.

Increased vascular inflammatory signaling with age appears to be linked to the age-related increase in oxidative stress. The age-associated development of a pro-inflammatory vascular endothelial phenotype in rat coronary arteries is associated with a “pro-oxidant” state characterized by increased gene and protein expression of NADPH oxidase, increased superoxide and peroxynitrite concentrations, and impaired EDD (19, 20). Plasma markers of oxidative stress are increased in older adults free of clinical disease and major CVD risk factors

(5, 31). Furthermore, expression of nitrotyrosine, a marker of oxidative modification, is greater in human vascular endothelial cells from older vs. young men (25). ROS production (e.g., superoxide and peroxynitrite formation) is increased in the vascular endothelium of aged animals (6, 9, 34, 38, 54, 74) and this is abolished by inhibition of NADPH oxidase-mediated superoxide production (9). Endothelial cells collected from human arteries and veins indicate an increased protein expression of NADPH oxidase with age (25). Antioxidant enzyme concentrations in the plasma of older humans (60) and the vasculature of aged animals (9, 22, 34, 74) generally are unchanged or reduced compared with younger subjects despite greater ROS production. Thus, ROS production increases with age without an associated (compensatory) increase in antioxidant defenses leading to oxidative stress.

In humans, the influence of oxidative stress in suppressing EDD can be determined by infusion of supra-physiological concentrations of vitamin C, a potent antioxidant that scavenges superoxide anions (31, 45). Using this approach, it has been demonstrated that vitamin C infusion restores brachial artery FMD in older healthy sedentary men, while having no effect on young men (31). These observations in humans are consistent with experimental findings that pretreatment with superoxide dismutase (SOD), the primary antioxidant enzyme for scavenging superoxide in the vascular wall, restores EDD in senescent mice and rats (6). In addition, inhibiting NADPH oxidase in carotid arteries from older mice improves EDD (27). Taken together, these data suggest that vascular oxidative stress develops with age and that NADPH oxidase is a significant source of superoxide in the vascular wall with aging.

A key mechanism mediating impaired EDD with aging could be increased NF-κB activation by pro-inflammatory cytokines and ROS (73). NF-κB DNA binding activity increases with aging in aortic, cardiac, liver, kidney, and brain tissues of rodents (43, 49, 86). Healthy middle-aged and older adults have a greater vascular endothelial expression of NF-κB in the whole cell and nucleus (a marker of activity) compared with young adults (24, 25). Thus, even in the absence of risk factors for CVD, aging is associated with systemic and vascular

endothelial oxidative stress, activation of NF-κB-mediated transcription of pro-oxidant enzymes and pro-inflammatory molecules, development of chronic low-grade systemic and vascular inflammation and impaired EDD (19, 20, 73).

Habitual aerobic exercise and preserved EDD

In contrast to the age-associated reduction in EDD observed in sedentary adults, individuals who perform regular aerobic exercise demonstrate preserved EDD with aging (23, 31). An aerobic exercise intervention, such as 8 to 12-weeks of brisk walking, improves EDD in middle-aged and older men (23, 64), although post-menopausal women appear to be less responsive to an exercise intervention (64). However, the cellular and molecular mechanisms that mediate the beneficial effect of habitual aerobic exercise on EDD in older adults have not been established, including the possible roles of reduced systemic and vascular inflammation.

If habitual exercise preserves EDD with aging by suppressing vascular inflammatory signaling and/or oxidative stress, it is important to determine the local vascular endothelial mechanisms responsible. Acutely reducing oxidative stress with infusion of vitamin C restores EDD in sedentary middle-aged and older men, but has no effect on EDD in their habitually exercising peers (31, 70, 71). Similarly, old mice given access to running wheels have lower aortic expression and activity of NADPH oxidase and expression of nitrotyrosine compared with old cage control mice (27). In the exercising old mice, NADPH oxidase inhibition does not affect EDD, indicating that old exercising mice do not have NADPH oxidase-mediated suppression of EDD. These observations suggest that older habitually exercising adults have reduced oxidative stress-induced suppression of EDD. In contrast to the evidence described above on the role of oxidative stress, presently there is no information in animals or humans on the effects of regular aerobic exercise on vascular inflammatory signaling with aging.

In conclusion, healthy middle-aged and older adults have impaired EDD and this is caused by reduced NO bioavailability and increased oxidative stress. Furthermore, healthy

middle-aged and older adults who participate in habitual aerobic exercise have preserved EDD as a result of reduced oxidative stress. However, the role of inflammatory signaling in the impaired EDD with sedentary aging and the preserved EDD with aerobic exercise is unknown.

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Chapter IV

Vascular Endothelial Function is Related to White Blood Cell Count and Myeloperoxidase Among Healthy Middle-Aged and Older Adults

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Abstract

Endothelium-dependent dilation (EDD) is impaired with aging, but there is significant variability among healthy middle-aged and older adults. We tested the hypothesis that EDD is related to white blood cell (WBC) count in healthy men and women aged 55–75 years (n=48) who have a WBC count within the clinically normal range. The peak forearm blood flow (FBF) response to intra-brachial artery infusion of acetylcholine was inversely related to WBC count (r=-0.38, P=0.004) and was 34% smaller in subjects with higher vs. lower WBC count (>vs.< median of $5.0x10^9$ cells/L, P=0.001). Vascular smooth muscle responsiveness to nitric oxide (NO, peak FBF response to sodium nitroprusside) was inversely related to WBC count (r=-0.30, P=0.02), but did not fully explain the associations with EDD. Inhibition of NO with N^G-monomethyl-L-arginine reduced EDD in subjects with lower (-56%, P=0.01), but not higher WBC count. Tetrahydrobiopterin selectively improved EDD in subjects with higher WBC count (+35%, P=0.01) by increasing NO bioavailability. EDD was related (P<0.05) to neutrophil, eosinophil and monocyte, but not lymphocyte or basophil counts. Myeloperoxidase, which is secreted by neutrophils and monocytes, consumes NO and produces molecules that oxidize tetrahydrobiopterin, was inversely related to EDD (r=-0.35, P=0.02) and was 42% higher in subjects higher WBC count (P=0.02). No other factors contributed to the relation between EDD and WBC count. Among healthy middle-aged and older adults, impaired EDD is related to higher neutrophil, eosinophil and monocyte-based WBC count mediated by reduced responsiveness to NO and increased myeloperoxidase-associated reductions in tetrahydrobiopterin and NO bioavailability.

Introduction

Because age is the major risk factor for cardiovascular diseases (CVD), middle-aged and older adults are at elevated risk for CVD in the absence of other conventional risk factors (11). Much of this increased risk is related to vascular endothelial dysfunction, a key feature of which is an impaired ability of peripheral arteries to dilate in response to a pharmacological or flow-induced stimulus (2, 11). Endothelial dysfunction, characterized by impaired endothelium-dependent dilation (EDD), is a predictor of future CVD-related events in older adults without clinical disease at baseline (22, 30).

EDD varies widely even among healthy middle-aged and older adults (3, 26). However, the factors that explain this inter-individual variability are not well understood. One such factor may be white blood cell (WBC) count. Within the normal clinical range, higher WBC count is associated with increased risk of future CV events (9, 14). Although only limited data are available, WBC count is inversely related to EDD among patients with clinical diseases such as type 2 diabetes and hypertension, and in smokers (8, 12, 29). It is unknown if EDD is related to WBC count among non-smoking, unmedicated middle-aged and older adults without chronic disease.

Little is known about the mechanisms that may link WBC count to EDD. In patients with type 2 diabetes, a higher WBC count is associated with a reduced dilation in response to the nitric oxide (NO) donor glyceryl trinitrate (29). This suggests that vascular smooth muscle responsiveness to NO, the major dilating molecule produced by the endothelium, may be reduced in patients with a higher WBC count. Aging generally is associated with reduced vascular NO bioavailability (26), in part as a result of reduced bioactivity of tetrahydrobiopterin (10), an essential co-factor for NO production by endothelial NO synthase (23). It is possible that middle-aged and older adults with higher WBC count may have greater impairments in EDD because of reduced tetrahydrobiopterin-mediated NO production and bioavailability.

Finally, the types of WBCs responsible for an association between total WBC count and EDD is important to establish and may have implications regarding the mechanisms involved. For example, myeloperoxidase is a peroxidase synthesized by neutrophils and monocytes that directly consumes NO and produces reactive oxygen species that oxidize tetrahydrobiopterin, collectively resulting in reduced NO bioavailability (1, 7). Higher circulating concentrations of myeloperoxidase are associated with impaired EDD in patients with rheumatoid arthritis (13) and cardiovascular disorders (27), but its relation to WBC count and EDD in middle-aged and older adults without chronic disease is unknown.

In the present study, we tested the hypothesis that EDD is inversely related to WBC count among non-smoking, unmedicated middle-aged and older adults free of chronic disease. To do so, we first examined the relation between WBC count and EDD within an overall sample of healthy adults aged 55-75 years. We then determined if EDD differed in groups of middle-aged and older adults with lower vs. higher WBC count compared to a reference group of young controls. We also determined which types of WBCs were related to EDD and gained insight into the potential mechanisms by which higher WBC count may be associated with impaired EDD.

Methods

Subjects. For the primary sample, data were obtained from 48 men and women aged 55–75 years. The subjects were divided into two equal groups based on the median WBC count (5.0 x 10^9 cells/L). Reference data for EDD and NO responsiveness were included on a group of healthy young adult controls (18-35 years; n=17, 13M). Subjects were free of clinical CVD, diabetes and other chronic diseases as assessed by medical history, physical examination, blood chemistries, ECG and blood pressure responses to incremental treadmill exercise performed to volitional exhaustion. Subjects were nonsmokers, not regularly exercising, not taking medications and refrained from dietary supplements for 4 weeks prior to the study. No subjects had an abnormally high WBC count (>10.0 x10^9 cells/L) that would

indicate an acute inflammatory response. All procedures were approved by the Human Research Committee of the University of Colorado at Boulder. The nature, benefits, and risks of the study were explained to the volunteers and their written informed consent was obtained prior to participation.

Procedures. All measurements were performed at the University of Colorado at Boulder Clinical and Translational Research Center after a 12-h fast and a 24-h abstention from alcohol and physical activity.

Subject characteristics. Arterial blood pressure was measured over the brachial artery during seated rest using a semiautomated device (Dinamap Pro 100, GE Health Care, Waukesha, WI). Fasting plasma metabolic factors were determined by the Clinical and Translational Research Center core laboratory using standard assays. White blood cell count was measured by standard Coulter counter technique (Beckman Coulter Ac·T 5diff CP, Fullerton, CA). ELISA was used to measure serum concentrations of myeloperoxidase (Prognostix, Cleveland, OH), oxidized low density lipoprotein (LDL)(ALPCO, Salem, NH), tumor necrosis factor (TNF)-α and interleukin (IL)-6 (R&D Systems, Minneapolis, MN). C-reactive protein was measured using a high-sensitivity Chemistry Immuno Analyzer (AU400e, Olympus America, Center Valley, PA).

Vasodilatory responses. Forearm blood flow (FBF) responses to incremental intra-brachial artery infusion of acetylcholine (1.0, 2.0, 4.0, and 8.0 μg·100 ml/forearm volume/min) (i.e., EDD) and sodium nitroprusside (0.5, 1.0, and 2.0 μg·100 ml/forearm volume/min) were measured in the experimental (nondominant) and the control (dominant) forearm of all subjects using strain-gauge venous occlusion plethysmography (Hokanson, Bellevue, WA) as described previously (5, 6, 19). The contribution of NO to the FBF responses to acetylcholine was determined in a subset of subjects (lower WBC count: n=4, 2m/2f; higher WBC count: n=4, 3m/1f) by co-infusing N^G-monomethyl-L-arginine (L-NMMA, Clinalfa AG, Bubendorf, Switzerland, 5 mg/min, 10-min loading dose) into the brachial artery during the incremental

infusion of acetylcholine. The role of tetrahydrobiopterin bioactivity in the FBF responses to acetylcholine and its NO component was determined in a subset of subjects (lower WBC count: n=11, 4m/7f; higher WBC count: n=12, 5m/7f) by co-infusing tetrahydrobiopterin (Clinalfa AG, 500 μg/min, 10-min loading dose) into the brachial artery during the incremental infusion of acetylcholine in the absence and presence of L-NMMA.

Data analysis. Statistical analyses were performed with SPSS (version 17.0.2; Chicago, IL). Pearson correlation analysis was used to assess bivariate relations of interest and multivariate analysis was used to determine the effect of additional factors on those relations. Differences in subject characteristics were determined by t-test for independent sample comparisons. The FBF responses to incremental doses of acetylcholine and sodium nitroprusside were analyzed by repeated-measures ANOVA. ANCOVA was used to determine the effects of an outside factor on group differences in primary outcome variables. Statistical significance for all analyses was set at $P < 0.05$. Values are mean ± SE.

Results

Subject Characteristics

Characteristics for the lower and higher WBC count subject groups are presented in Table 1. WBC count was 50% greater in the group with higher WBC count (P<0.001). Values for risk factors were within the normal clinical ranges for both groups. There were no group differences in age, blood pressure or plasma lipids. The group with higher WBC count had higher fasting blood glucose (P=0.004). Circulating concentrations of C-reactive protein tended to be greater (P=0.06) in the subjects with higher WBC count, whereas IL-6, TNF-α and oxidized LDL were not different in the two groups.

Table 1: Group Subject Characteristics

Variable	Lower WBC Count	Higher WBC Count
N (m/f)	24 (13/11)	24 (13/11)
White blood cell count, 10^9 cells/L	4.1 ± 0.1	6.0 ± 0.2 *
Age, yr	63 ± 1	63 ± 1
Body mass index, kg/m^2	26 ± 1	28 ± 1
Waist:Hip Ratio	0.85 ± 0.02	0.86 ± 0.02
Systolic blood pressure, mm Hg	123 ± 3	121 ± 2
Diastolic blood pressure, mm Hg	75 ± 2	75 ± 2
Total cholesterol, mg/dl	210 ± 5	198 ± 6
LDL-Cholesterol, mg/dl	117 ± 5	115 ± 5
HDL-Cholesterol, mg/dl	60 ± 3	53 ± 3
Triglycerides, mg/dl	110 ± 10	121 ± 8
Fasting blood glucose, mg/dl	88 ± 1	95 ± 2 *
C-reactive protein, mg/L	1.0 ± 0.3	1.7 ± 0.3
Interleukin-6, pg/ml	1.2 ± 0.2	1.4 ± 0.2
Tumor necrosis factor-α, pg/ml	1.5 ± 0.2	1.7 ± 0.2
Myeloperoxidase, pmol/L	327 ± 30	465 ± 52 *
Oxidized LDL, U/L	60 ± 3	59 ± 3

Data are mean±SE. *$p<0.05$ vs. lower WBC count.

Vasodilatory Responses

***EDD*.** Among all middle-aged and older subjects, the peak FBF response to acetylcholine was inversely related to WBC count (r=-0.38, P=0.004). When subjects were divided into groups based on WBC count, the FBF responses to acetylcholine were smaller in subjects with a higher WBC count, with a peak vasodilatory response 34% less than the group with a lower WBC count (P=0.02, Figure 1A). The FBF responses to acetylcholine in the groups with higher and lower WBC count were smaller (P=0.006) and not different (P=0.55), respectively, compared with young adult controls (age=25±1 yr, WBC count=5.4 x 10^9 cells/L). Thus, EDD is impaired in middle-aged and older adults with a higher WBC count compared with their peers with a lower WBC count and young adults.

Sensitivity to NO. Among all subjects, the peak FBF response to sodium nitroprusside was inversely related to WBC count (r=-0.30, P=0.02). Consistent with this relation, subjects with a higher WBC count had smaller FBF responses to sodium nitroprusside, with a peak response 18% less than the group with a lower WBC count (P=0.04, Figure 1B). The FBF responses to sodium nitroprusside in the groups with higher and lower WBC count were smaller (P=0.007) and not different (P=0.36), respectively, compared with the young adult controls. These observations suggest that vascular smooth muscle relaxation and vasodilation in response to NO is reduced in middle-aged and older adults with a higher WBC count.

To determine if differences in vasodilatory responsiveness to NO explained the relation between EDD and WBC count, we performed a multivariate analysis in the overall group. The peak FBF response to sodium nitroprusside contributed to the relation between WBC count and peak FBF response to acetylcholine (P<0.001). However, the WBC count-peak FBF response to acetylcholine association remained significant after adjustment for the peak FBF response to sodium nitroprusside (partial correlation coefficient: r=-0.27, P=0.04). Similar results were obtained by ANCOVA with the group comparisons. These results indicate that reduced

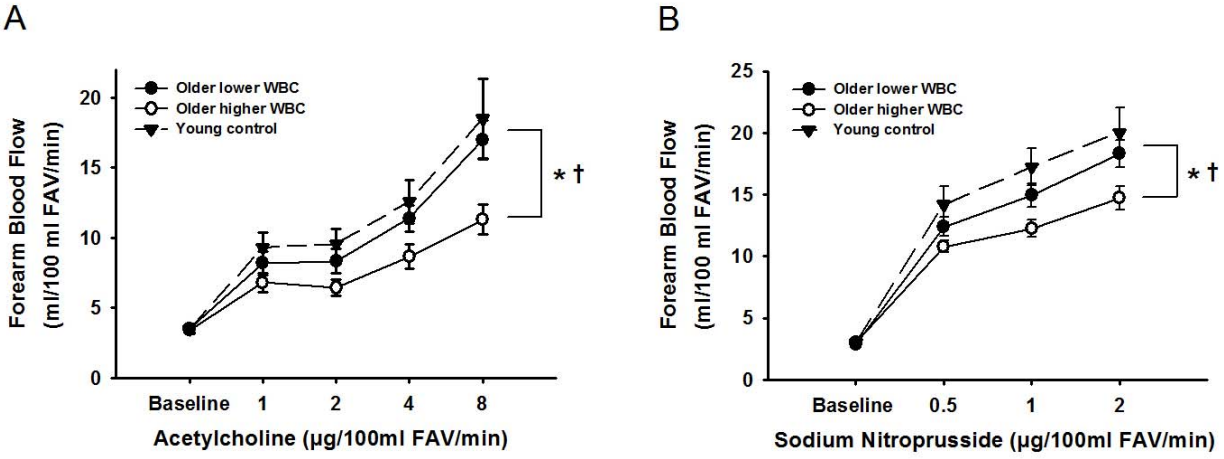

Figure 1: White blood cell (WBC) count, endothelium-dependent dilation (EDD) and nitric oxide (NO) responsiveness. Middle-aged and older adults with a higher WBC count had (A) impaired EDD, assessed by the peak forearm blood flow (FBF) response to acetylcholine (ACh), and (B) impaired NO responsiveness, assessed by the peak forearm blood flow (FBF) response to sodium nitroprusside (SNP), when compared with ma/o with a lower WBC count and young controls. *P<0.05 vs. lower WBC count group. †P<0.05 vs. young controls. FAV indicates forearm volume.

sensitivity to NO contributes to, but does not completely explain, the greater impairments in EDD in the subjects with a higher compared with lower WBC count.

Role of NO Bioavailability. Inhibition of NO production with L-NMMA reduced the FBF response to acetylcholine in subjects with a lower WBC count (P=0.01), but did not significantly affect the response in those with a higher WBC count (P=0.30) (Figure 2). As a result, there were no differences in the FBF responses to acetylcholine between the groups in the absence of NO production (P=0.48). This indicates that the greater impairment in baseline EDD in the subjects with a higher WBC count is mediated by reduced NO bioavailability.

Role of Tetrahydrobiopterin. Infusion of tetrahydrobiopterin improved the FBF responses to acetylcholine in subjects with a higher WBC count (P=0.01), but had no effect in the subjects with a lower WBC count (P=0.41, Figure 3). This suggests that the impaired FBF responses to acetylcholine in middle-aged and older adults with a higher WBC count are mediated, at least in part, by reduced vascular bioactivity of tetrahydrobiopterin.

Inhibition of NO production using L-NMMA reduced the FBF responses to co-infusion of acetylcholine and tetrahydrobiopterin in both groups (both $P \leq 0.008$, Figure 3), abolishing the vasodilatory-enhancing effects of tetrahydrobiopterin in the subjects with a higher WBC count. These findings provide evidence that increased NO bioavailability was the mechanism for the tetrahydrobiopterin-mediated improvements in EDD in middle-aged and older adults with a higher WBC count.

WBC Subpopulations. Among all subjects, neutrophil count demonstrated the strongest relation to the peak FBF response to acetylcholine (r=-0.38, P=0.005, Figure 4A). Eosinophil and monocyte count also were related to the peak FBF response to acetylcholine (Table 2). Basophil and lymphocyte count were not related to the peak FBF response to acetylcholine (Table 2). These findings indicate that neutrophils and, to a lesser extent, eosinophils and monocytes, were the subpopulations of WBCs that contributed most to the relation between EDD and WBC count.

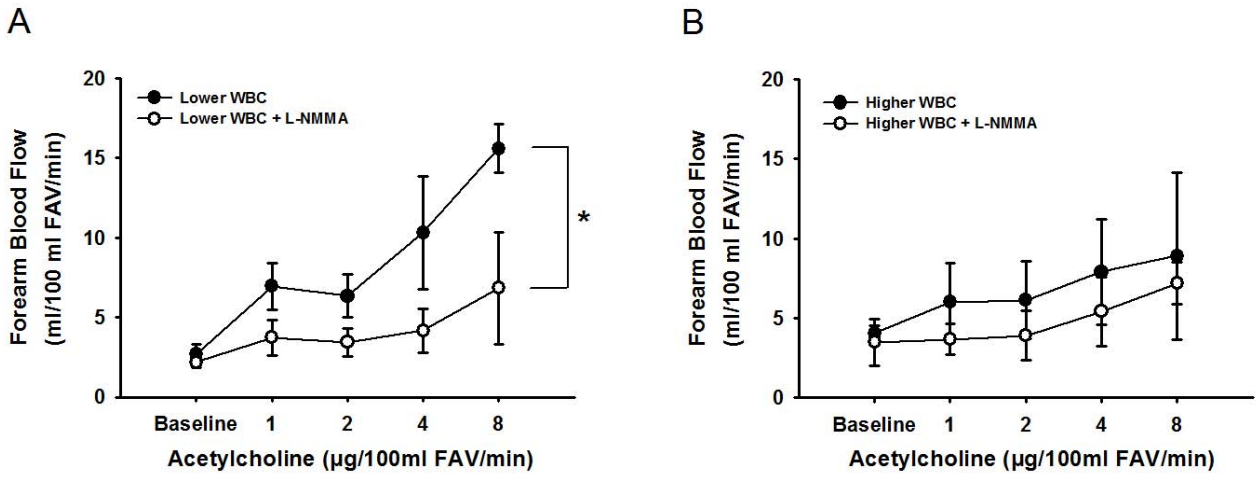

Figure 2: Role of nitric oxide (NO) bioavailability in white blood cell (WBC) count-endothelium-dependent dilation association. The forearm blood flow response to acetylcholine was reduced with co-infusion of the NO inhibitor N^G-monomethyl-L-arginine (L-NMMA) in subjects with a lower WBC count (A), but did not change in subjects with a higher WBC count (B) such that there were no group differences in the absence of NO production. *P<0.05 vs. acetylcholine alone. FAV indicates forearm volume.

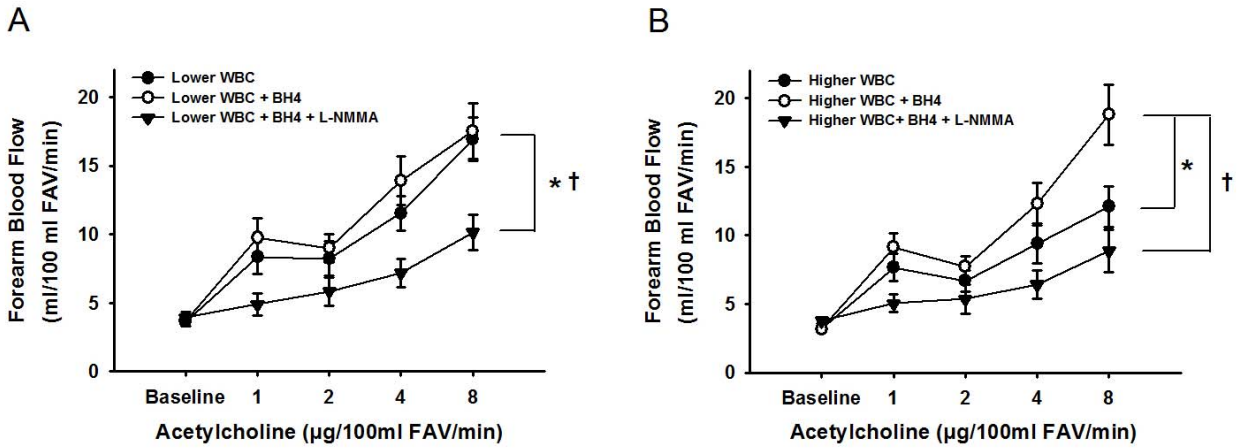

Figure 3: Role of tetrahydrobiopterin (BH_4) modulation of nitric oxide (NO) bioavailability in white blood cell (WBC) count-endothelium dependent dilation association. The forearm blood flow response (FBF) to acetylcholine was enhanced during co-infusion of BH_4 in subjects with higher WBC count (B), but did not change for subjects with lower WBC count (A) such that there were no group differences when BH_4 was supplemented. Co-infusion with the NO inhibitor N^G-monomethyl-L-arginine (L-NMMA) during acetylcholine and BH_4 infusion resulted in a decline in FBF response in both groups (A and B) such that there were no group differences in effects of BH_4 in the absence of NO production. *P<0.05 vs. acetylcholine alone. †P<0.05 vs. acetylcholine with BH_4. FAV indicates forearm volume.

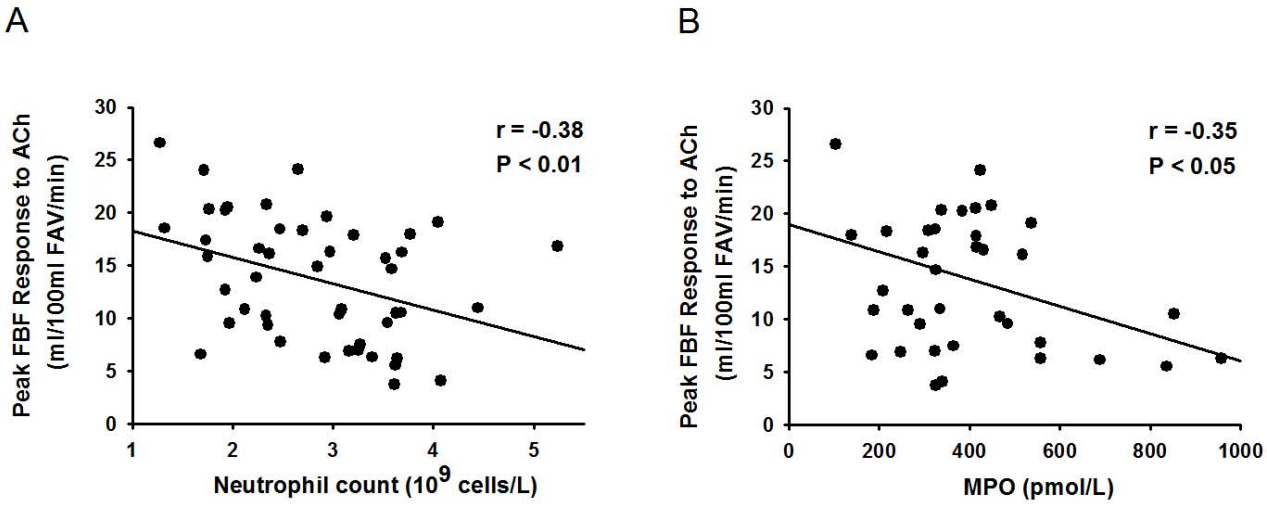

Figure 4: Neutrophil count, myeloperoxidase and endothelium-dependent dilation (EDD). EDD assessed by the peak forearm blood flow (FBF) response to acetylcholine (ACh) was inversely related to (A) neutrophil count and (B) serum myeloperoxidase concentrations. FAV indicates forearm volume.

Table 2: Associations Between Types of WBCs and EDD

White blood cell Differential Count (10^9 cells/L)	Peak FBF Response to ACh (ml/100ml FAV/min)	
	r	P
Neutrophil	-0.38	<0.01
Eosinophil	-0.30	0.02
Monocyte	-0.27	0.03
Basophil	-0.20	0.09
Lymphocyte	-0.15	0.15

FBF, forearm blood flow; ACh, acetylcholine; FAV, forearm volume.

Role of Myeloperoxidase. Serum myeloperoxidase was 42% greater in the subjects with a higher WBC count (P=0.01) and was inversely related to the peak FBF response to acetylcholine in the overall group (r=-0.35, P=0.02, Figure 4B). Multivariate analysis indicated that myeloperoxidase contributed to the relation between WBC count and peak FBF response to acetylcholine (P=0.05), but WBC count remained a significant predictor of the peak acetylcholine response after adjustment for myeloperoxidase (partial correlation coefficient: r=-0.33, P=0.02). Similar results were obtained with ANCOVA in the group comparisons. These results suggest that myeloperoxidase contributes to, but does not completely explain, the relation between WBC count and EDD.

Relations to Other Factors. The peak FBF response to acetylcholine was inversely related to plasma C-reactive protein (r=-0.34, P=0.02). However, accounting for C-reactive protein with multivariate analysis did not influence the relation between the peak FBF response to acetylcholine and WBC count (partial correlation coefficient: r=0.39, P=0.005). These findings indicate that the greater impairment in EDD in the subjects with higher WBC count was not related to their greater C-reactive protein concentrations.

Peak FBF responses to acetylcholine were not related to measures of body fatness, blood pressure, plasma lipids, fasting blood glucose levels or serum concentrations of IL-6, TNF-α or oxidized LDL. Collectively, these results indicate that WBC count was the best predictor of EDD among all subject characteristics and circulating factors.

Other Relations to WBC Count

WBC count was related to myeloperoxidase (r=0.33, P=0.02), C-reactive protein (r=0.32, P=0.02) and fasting blood glucose (r=0.45, P=0.001).

Discussion

Our results provide evidence that acetylcholine-induced EDD, a common expression of vascular endothelial function and predictor of future CVD risk, is inversely related to WBC count among non-smoking, unmedicated middle-aged and older men and women without clinical disease. Within the clinically normal range of WBC count, a group with higher WBC concentrations had impaired EDD compared with their peers with a lower WBC count and young adults. WBC count was a stronger predictor of EDD than other subject characteristics and circulating factors, including other markers of inflammation such as serum C-reactive protein. The inverse relation between WBC count and EDD observed among middle-aged and older adults is consistent with and extends previous observations in patients with diabetes and hypertension and in smokers (8, 12, 29). We also determined that this overall relation with WBC count was due to significant associations between EDD and neutrophil (strongest), eosinophil and monocyte counts. Finally, our findings indicate that the mechanisms contributing to the WBC count-EDD relation include impaired vascular smooth muscle responsiveness to NO and tetrahydrobiopterin-linked reductions in NO bioavailability associated with increases in circulating myeloperoxidase.

Responsiveness to NO. We found that vasodilation to the NO donor sodium nitroprusside was impaired in subjects with a higher WBC count, consistent with previous findings in patients with diabetes (29). A reduced vasodilatory response to sodium nitroprusside reflects a decrease in NO signaling in vascular smooth muscle cells, most likely as a result of impaired cyclic GMP signaling (16). However, in the present study vasodilatory responsiveness to sodium nitroprusside did not fully explain the relation between EDD and WBC count among individuals or between groups, suggesting that other mechanisms are involved.

NO and Tetrahydrobiopterin Bioavailability. In the present study, the greater impairment in EDD in subjects with higher WBC count was mediated by reduced NO

bioavailability because inhibition of NO production using L-NMMA abolished baseline group differences in EDD.

One factor contributing to NO production is tetrahydrobiopterin, an essential co-factor for NO synthase. Infusion of tetrahydrobiopterin improves EDD on average in middle-aged and older adults (10), suggesting that reduced bioactivity of tetrahydrobiopterin contributes to age-associated reductions in EDD, at least in some individuals. In the present study, infusion of tetrahydrobiopterin increased EDD in the group with a higher WBC count, but had no effect in the group with a lower WBC count. This indicates that among healthy non-smoking middle-aged and older adults, a higher WBC count is associated with reduced vascular tetrahydrobiopterin bioactivity.

Reduced tetrahydrobiopterin bioactivity should limit production of NO by the vascular endothelium via "uncoupling" of endothelial NO synthase (15), with a consequent reduction in NO bioavailability. Consistent with this idea, we found that co-infusion of L-NMMA abolished the selective tetrahydrobiopterin-associated improvement in EDD in the subjects with a higher WBC count, resulting in similar responses in the two groups. These data support the concept that the greater impairment in EDD in middle-aged and older adults with a higher WBC count is mediated by reduced tetrahydrobiopterin bioactivity-dependent decreases in NO bioavailability.

Because tetrahydrobiopterin restored EDD in the subjects with a higher WBC count, and reduced NO responsiveness was found to contribute to impaired EDD in these subjects, it is possible that tetrahydrobiopterin restored EDD in part by increasing responsiveness to NO. We did not determine the effects of tetrahydrobiopterin on the FBF responses to sodium nitrioprusside in the present study and, therefore, are unable to provide direct insight into this possibility.

Types of WBCs Involved. Our results indicate that the inverse relation between EDD and total WBC count was due to significant inverse relations between EDD and neutrophils, eosinophils and monocytes. Among these cell types, the strongest relation to EDD was with

neutrophils, a cell population that is a strong predictor of future CV events (20, 25, 28). In contrast, EDD was not related to basophils or lymphocytes. The weakest relation to EDD was with lymphocytes, which, among WBC types, are the weakest predictors of CVD risk (20, 25).

Myeloperoxidase. Because neutrophils produce the majority of circulating myeloperoxidase (24) and serum myeloperoxidase is a predictor of EDD in patients with clinical disease (13, 27), myeloperoxidase concentrations and their relation to EDD were assessed in the present study. We found that serum myeloperoxidase was inversely related to EDD in our overall sample and contributed to the relation between EDD and WBC count. Consistent with this, serum myeloperoxidase concentrations were significantly greater in the subjects with higher WBC count. Myeloperoxidase reduces NO bioavailability by a number of mechanisms including direct consumption of NO and production of reactive oxygen species that oxidize tetrahydrobiopterin to its inactive form, which, in turn, uncouples endothelial NO synthase (1, 7). As such, it is possible that the greater serum myeloperoxidase in subjects with a higher WBC count contributed to their impaired EDD by reducing NO bioavailability via increased consumption of NO and decreased tetrahydrobiopterin bioactivity and NO production.

Circulating Inflammatory Proteins. We found that C-reactive protein, an acute phase protein and most commonly used clinical marker of systemic inflammation, was greater in subjects with a higher WBC count, with concentrations corresponding to a moderately increased risk of CVD (17). In contrast, serum concentrations of the cytokines IL-6 and TNF-α did not differ between groups. However, controlling for C-reactive protein concentration did not alter the relation between EDD and WBC count among individuals. Thus, markers of systemic inflammation do not obviously explain the relation between EDD and WBC count in present study.

Local Interactions. The influence of WBC count on EDD may be mediated in part by local interactions with the vascular wall. WBCs are immune cells that constantly interact with the endothelial cell layer via rolling, adhesion and infiltration into the vascular wall (18, 21).

Upon interacting with the vascular wall, WBCs can produce and release reactive oxygen species and cytokines, which could, in turn, influence gene and protein expression, intra-cellular signaling and vasodilatory responsiveness (4). Thus, the modulatory influence of WBC count on EDD in middle-aged and older adults may be, in part, the result of physical or chemical interactions with the vascular endothelium.

***Perspectives*.** Our results demonstrate that EDD is inversely related to WBC count among non-smoking middle-aged and older adults without clinical disease. Thus, WBC count appears to be a key factor that influences EDD and contributes to its variability in this group. Importantly, our findings show that the mechanisms linking WBC count to EDD in these subjects involve decreased vascular smooth muscle sensitivity to NO and tetrahydrobiopterin-associated reductions in NO bioavailability. The relation between EDD and WBC count is due to inverse relations between EDD and selective populations of WBCs, with neutrophils having the strongest association. Increased myeloperoxidase produced by neutrophils could be an important mechanism for reduced tetrahydrobiopterin bioactivity and NO bioavailability. Indeed, WBC count and serum myeloperoxidase were more strongly related to EDD than any other subject characteristic or circulating factor in the present study. Overall, our findings may have important clinical implications for identifying and treating middle-aged and older adults who are at greater risk for vascular endothelial dysfunction and CV events.

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Chapter V

Absence of nuclear factor-κB-mediated suppression of vascular endothelial function in middle-aged and older adults who habitually perform aerobic exercise

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Abstract

Adults who habitually perform aerobic exercise demonstrate preserved endothelium-dependent dilation (EDD) with age. We hypothesized that reduced endothelial signaling by nuclear factor-κB (NF-κB), a key pro-inflammatory transcription factor, is a mechanism for this preservation of EDD. In young sedentary (n=23, 18M/5F) and middle-aged and older (MA/O) sedentary (n=23, 19M/4F) and MA/O aerobic exercise-trained (n=23, 19M/4F) healthy adults, EDD was assessed by brachial artery flow-mediated dilation (FMD). MA/O exercise-trained adults had a greater FMD than their sedentary peers (7.3±0.4% vs. 4.3±0.5%, $P<0.05$) and values not different from young adults (6.9±0.6%, $P>0.05$). Endothelial cell expression of NF-κB p65 was lower in MA/O exercise-trained and young vs. MA/O sedentary adults (0.41±0.3 and 0.33±0.02 vs. 0.53±0.06, $P<0.05$). In a sub-group of MA/O sedentary and exercise-trained subjects, oral salsalate was used to inhibit NF-κB signaling. Salsalate treatment reduced endothelial cell NF-κB p65 expression by 24% in sedentary MA/O ($P<0.05$), but had no effect in exercise-trained MA/O adults ($P>0.05$). FMD improved by 74% in sedentary ($P<0.001$), but did not change in exercise-trained individuals ($P>0.05$) with salsalate. In sedentary MA/O adults, vitamin C infusion improved FMD by 32% during placebo ($P<0.001$), but had no affect during salsalate treatment ($P>0.05$). In exercise-trained MA/O subjects, vitamin C infusion did not change FMD during either treatment condition ($P>0.05$). Endothelium-independent dilation was not different between conditions. Salsalate treatment reduced endothelial expression of nitrotyrosine by 18% and NADPH oxidase p47 by 30% in sedentary MA/O subjects ($P<0.05$), but had no effect in exercise-trained adults ($P>0.05$). Thus, reduced NF-κB signaling, and associated oxidative stress, is a key mechanism for preserved EDD with habitual aerobic exercise in MA/O adults.

Introduction

Vascular endothelial dysfunction, characterized by impaired endothelium-dependent dilation (EDD), is a predictor of future cardiovascular events (21, 28, 29). Impairments in EDD are observed in middle-aged and older compared with young healthy sedentary adults (5, 12). Individuals who habitually perform aerobic exercise demonstrate a preserved EDD with age (11-13, 22), but the mechanisms for this preservation are not understood.

Pro-inflammatory signaling is associated with atherosclerotic cardiovascular disease and impaired EDD (16, 25). In endothelial cells, the expression and activity of nuclear factor-κB (NF-κB), a key pro-inflammatory transcription factor, increases with age (8, 9). We have previously shown that endothelial NF-κB signaling can be inhibited with short-term oral salsalate (non-acetylated salicylate) administration (20). Salsalate administration improves EDD in overweight and obese adults with characteristics of the metabolic syndrome compared to placebo treatment (20), indicating a tonic suppression of EDD by pro-inflammatory signaling in these individuals. Because habitual aerobic exercise often is associated with lower circulating markers of inflammation in middle-aged and older adults (6, 24) it is believed to exert anti-inflammatory effects (3, 14). As such, it is possible that regular aerobic exercise acts to preserve vascular endothelial function with aging by preventing age-associated increases in NF-κB-related suppression of EDD.

Inflammatory signaling increases the production of reactive oxygen species, particularly by activating oxidant enzymes, such as NADPH oxidase (1, 17). Nitrotyrosine, a marker of oxidative damage, increases in endothelial cells with age and relates to the expression of endothelial NF-κB (9). The expression of NADPH oxidase subunits also increase with age in endothelial cells (9). Healthy sedentary middle-aged and older adults demonstrate tonic oxidative-stress mediated suppression of EDD compared with young adults, as indicated by improvements in EDD to acute infusion of antioxidant vitamin C (12). This oxidative stress-mediated suppression of EDD is absent in middle-aged older adults who habitually perform

aerobic exercise (12). However, it is unknown if the potential anti-inflammatory effects of regular aerobic exercise on vascular endothelial function with aging are mediated by reduced vascular oxidative stress.

The goal of this study was to determine the mechanistic role of reduced endothelial NF-κB signaling in the enhanced EDD observed in aerobic exercise-trained compared with sedentary middle-aged and older adults. We hypothesized that aerobic exercise-trained middle-aged and older adults would have greater EDD and less vascular endothelial expression of NF-κB than their sedentary peers, and similar to that observed in a young reference group. In addition, aerobic exercise-trained middle-aged and older adults would have less NF-κB-associated suppression of EDD compared with their sedentary peers. We also hypothesized that the lower tonic NF-κB-related suppression of EDD in aerobic exercise-trained vs. sedentary middle-aged and older adults would be associated with a corresponding reduction in oxidative stress-mediated inhibition of vascular endothelial function.

Methods

Subjects. Forty-six healthy middle-aged and older (50-77 years) men and women were studied: 23 sedentary and 23 aerobic exercise-trained. Reference data for baseline EDD and NF-κB expression were included for a group of young (n=23, 18-35 years) healthy sedentary adult controls. Sedentary was defined as no regular exercise (<30 min/day, <2 days/week) during the previous 2 years. Aerobic exercise-trained was defined as more than 4 sessions/week of vigorous aerobic-endurance exercise. All of the subjects had total cholesterol <240 mg/dl, LDL-cholesterol <160 mg/dl, resting blood pressure <140/90 mmHg, fasting blood glucose <126 mg/dl, body mass index (BMI) <35 kg/m^2 and were nonsmokers and free of clinical diseases as assessed by medical history, physical examination, blood chemistry, and resting and exercise ECG. Subjects were not taking medications and had refrained from antioxidants (e.g., vitamins C and E) and aspirin within 2 weeks of the study. All procedures

were approved by the Institutional Review Board of the University of Colorado at Boulder. The nature, benefits and risks of the study were explained to the volunteers, and their written informed consent was obtained before participation.

Procedures. All measurements were performed at the University of Colorado at Boulder Clinical and Translational Research Center after a 12-hour fast and 24-hour abstention from alcohol and physical activity.

Subject Characteristics. Waist and hip circumferences and BMI were measured by anthropometry. Percent body fat was measured by dual-energy X-ray absorptiometry (DXA-GE; Lunar Corporation; software version 5.60.003, Madison, Wis). Maximal oxygen consumption was measured during incremental treadmill exercise using open-circuit spirometry as previously described (7). Arterial blood pressure was measured over the brachial artery during supine rest using a semi-automated device (Dinamap Pro 100, GE Health Care, Waukesha, WI).

Blood Analyses. Fasting plasma metabolic factors were determined by the Clinical and Translational Research Center core laboratory using standard assays. ELISA was used to measure serum interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α, R&D Systems, Minneapolis, MN). C-reactive protein (CRP) was measured using a high-sensitivity Chemistry Immuno Analyzer (AU400e, Olympus America, Center Valley, PA).

EDD and endothelium-independent dilation. Duplex ultrasonography was used to assess EDD (brachial artery flow-mediated dilation, FMD) and endothelium-independent dilation (dilation to glyceryl trinitrate, GTN) in the supine position, as previously described by our laboratory (12, 13). Briefly, an ultrasound probe was clamped 3-6 cm proximal to the antecubital crease. After obtaining a baseline image, reactive hyperemia was produced by inflation of a blood pressure cuff placed on the upper forearm distal to the antecubital fossa for 5 min at 250 mmHg followed by a rapid deflation. Endothelium-independent dilation was assessed by measurement of brachial artery dilation in response to sublingual GTN (0.4 mg). Pulsed doppler signals were recorded at an angle of insonation of 68 degrees with a sample

volume the entire width of the artery as previously described (20). Time-averaged peak velocity was obtained from recording the first 10 velocity envelopes. Brachial artery peak hyperemic shear rate was calculated as 8 times (due to wide sample volume) the peak velocity immediately following 5 minutes of forearm occlusion, divided by occlusion diameter. FMD responses were expressed as relative (%) and absolute (mm) change from baseline diameter. During salsalate and placebo conditions (see below), FMD was measured first during saline infusion (control) and then during supraphysiological intravenous infusion of vitamin C as previously described (12). 0.06 g/kg fat-free mass of vitamin C was infused for 20 minutes, immediately followed by FMD measurements during a 0.02 g/kg fat-free mass vitamin C maintenance drip infusion.

Endothelial cell protein expression. The procedures used for collection of venous endothelial cells and measurement of protein expression by quantitative immunofluorescence have been described in detail previously by our laboratory (8, 9, 23). Two sterile J wires (Daig Corp, Minnetonka, Minn) were advanced into an antecubital vein (~4 cm beyond the tip of the catheter) and retracted through an 18-gauge catheter. The wires were then transferred to a dissociation buffer solution, and cells were recovered after a washing and centrifugation protocol. Collected cells were fixed with 3.7% formaldehyde and plated on poly-L-lysine–coated slides (Sigma Chemical, St. Louis, Mo) and then frozen at –80°C until analysis. Cells were rehydrated, and nonspecific binding sites were blocked with 5% donkey serum (Jackson Immunoresearch, West Grove, Pa). Cells were incubated with monoclonal antibodies for NF-κB p65 (Novus, Littleton, Colo), nitrotyrosine and NADPH oxidase p47phox (Abcam, Cambridge, Mass). Cells were then incubated with an Alexaflour 555 fluorescent secondary antibody (Invitrogen Corp, Carlsbad, Calif).

For analysis, slides were viewed with a fluorescence microscope (Eclipse 600, Nikon, Melville, NY), and cell images were captured digitally by a Photometrics CoolSNAPfx digital camera (Roper Scientific, Inc, Tucson, Ariz). Endothelial cells were identified by staining for von

Willebrand factor and nuclear integrity was confirmed with DAPI (4',6'-diamidino-2-phenylindole hydrochloride). Once endothelial cells with intact nuclei were identified, they were analyzed with Metamorph Software (Universal Imaging Corp, Downingtown, Pa). For an estimate of nuclear NF-κB p65, the nuclear regions of endothelial cells were identified by DAPI staining, overlaid on the same cell's Alexaflour 555 image and analyzed for fluorescent intensity of the Alexaflour within the DAPI region. Values for each protein are reported as a ratio of endothelial cell to human umbilical vein endothelial cell average pixel intensity. The technician was blinded to the identity of the subject and the experimental condition during the staining and analysis procedures. The reproducibility of our measurements of total and nuclear NF-κB was determined recently in 4 subjects in whom endothelial cells were collected at least 1 week apart (8). Reproducibility was defined as the absolute difference in endothelial cell mean fluorescent intensity in trial 1 versus trial 2 divided by average mean fluorescent intensity for the trials. Mean values (range) were as follows: total NF-κB 6.3% (4% to 8%) and nuclear NF-κB 7.1% (2% to 11%).

Salsalate administration. In a subset of sedentary (n=15, 11M/4F) and aerobic exercise-trained (n=13, 11M/2F) middle-aged and older adults, salsalate was administered similar to a previous protocol by our lab (20). Briefly, in a double-blind randomized crossover design, subjects were assigned oral doses of salsalate or placebo for 3 days prior to experimental testing. Serum salicylate was measured on the morning of days 2 and 3 and the day of experimental testing. Subjects received 2500 mg to 4500 mg of salsalate each day to result in a steady-state serum salicylate in the therapeutic range of 10 to 30 mg/dL. Doses were adjusted each day based on serum salicylate concentration to maintain salicylate in the therapeutic range without reaching toxicity (>30 mg/dl). For the 3 days prior to each experimental testing procedure (salsalate and placebo conditions), subjects received a standardized research diet prepared by the Clinical and Translational Research Center bionutritionist.

Data analysis. Statistical analyses were performed with PASW (version 18.0, Chicago, IL). Differences in subject characteristics between peer groups were assessed by t-tests for independent sample comparisons. Group differences in baseline EDD and protein expression were determined by one-way ANOVA. In the case of significant F values, *post hoc* analyses were performed using a Student-Newman-Keuls test. Differences within subject groups during salsalate vs. placebo were compared with paired t-tests. Linear regression was used to determine the effect of baseline subject characteristics and the change in circulating factors on the change in EDD with salsalate. Significance was set at $P<0.05$. Values are mean ± SE.

Results

Subject Characteristics. Characteristics for the sedentary and aerobic exercise-trained middle-aged and older men and women are presented in Table 1. There were no differences in age, blood pressure, total cholesterol, LDL-cholesterol or triglycerides between groups ($P>0.05$). Sedentary subjects had a greater BMI, percent body fat, waist:hip ratio, and fasting glucose and a lower VO_2max and HDL-cholesterol than the aerobic exercise-trained subjects ($P<0.05$).

EDD. Middle-aged and older sedentary subjects had ~40% lower brachial FMD compared with middle-aged and older exercise-trained subjects and the young reference group ($p<0.05$, Figure 1A and Table 2). Middle-aged and older exercise-trained subjects had a similar brachial FMD as the young reference group ($p>0.05$, Figure 1A and Table 2). These differences were not affected by baseline artery diameter, peak shear rate or endothelium-independent dilation (response to glyceryl trinitrate), as there were no differences between groups for these factors ($p>0.05$, Table 2). These results indicate that EDD is impaired with aging, but preserved with habitual aerobic exercise.

Endothelial cell NF-κB expression. Middle-aged and older sedentary subjects had a 36% and 55% greater endothelial cell NF-κB p65 protein expression in the whole cell when

Table 1: Subject characteristics

	Sedentary	Exercise-Trained
n (male/female)	23 (19/4)	23 (19/4)
Age (years)	61 ± 1	60 ± 1
Body mass index (kg/m^2)	27 ± 1	23 ± 1*
% Body fat	31 ± 1	18 ± 1*
Waist:Hip ratio	0.91 ± 0.02	0.86 ± 0.02 *
VO_2max (ml/kg/min)	30 ± 1	42 ± 2 *
Systolic blood pressure (mm Hg)	123 ± 3	119 ± 2
Diastolic blood pressure (mm Hg)	79 ± 1	76 ± 2
Total cholesterol (mg/dL)	193 ± 6	200 ± 6
LDL-cholesterol (mg/dL)	120 ± 5	122 ± 6
HDL-cholesterol (mg/dL)	49 ± 2	56 ± 2 *
Triglycerides (mg/dL)	119 ± 10	109 ± 9
Glucose (mg/dL)	92 ± 2	87 ± 2 *

Data are mean ± SE. VO_2max, maximal oxygen consumption. * P<0.05 vs. Sedentary

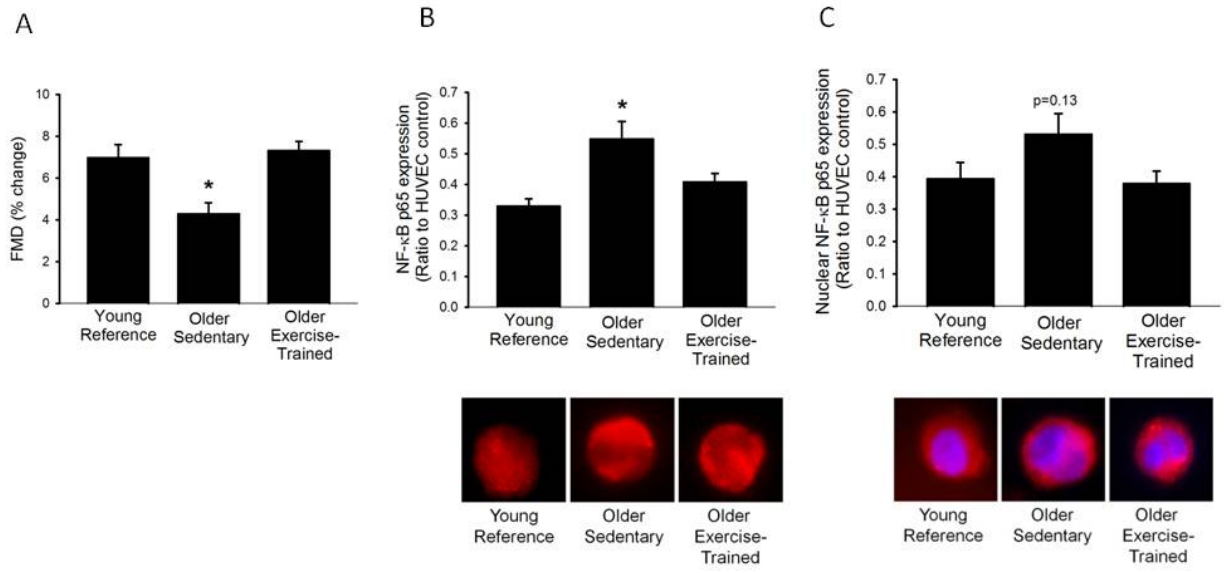

Figure 1. (A) Percent brachial flow-mediated dilation (FMD) and (B) whole cell and (C) nuclear endothelial protein expression of nuclear factor-κB (NF-κB) p65 in healthy middle-aged and older sedentary and aerobic exercise-trained adults and young reference group. Representative images of endothelial cells are shown below summary graphs. HUVEC: human umbilical vein endothelial cell. *P<0.05 vs. older exercise-trained and young reference. Values are mean ± SE.

Table 2: Brachial artery parameters

	Young Reference	Older Sedentary	Older Exercise-Trained
Brachial artery diameter (mm)	4.0 ± 0.1	4.0 ± 0.1	3.6 ± 0.1
FMD (mm change)	0.29 ± 0.03	0.16 ± 0.02 *	0.26 ± 0.01
Peak shear rate (1/s)	906 ± 84	811 ± 51	899 ± 76
GTN dilation (% change)	21 ± 3	23 ± 1	25 ± 1
GTN dilation (mm change)	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1

Data are mean ± SE. FMD, flow-mediated dilation; GTN, glyceryl trinitrate. * P<0.05 vs. young reference and older exercise-trained

compared with middle-aged and older exercise-trained subjects and the young reference group, respectively (P<0.05, Figure 1B). A similar pattern was observed when nuclear protein expression of NF-κB p65 (a marker of activity) was compared between groups (P=0.13, Figure 1C). Thus, with age in sedentary adults without clinical disease, endothelial cell NF-κB expression is increased and there are trends for increased activation. However, habitual aerobic exercise protected against the age-related increase in NF-κB expression and activity.

Inhibition of endothelial cell NF-κB by salsalate treatment. To determine if the preserved EDD with exercise training is a result of an absence of inflammatory signaling by NF-κB, we used oral salsalate to inhibit NF-κB in a subgroup of sedentary and aerobic exercise-trained middle-aged and older adults. Salsalate treatment resulted in serum salicylate concentrations in the therapeutic range for all subjects (>10 mg/dL). The final serum salicylate concentration was not different between sedentary and aerobic exercise-trained subjects (21.3±1.2 vs. 19.3±0.9 mg/dL, P = 0.24). Serum salicylate was undetectable in all subjects during the placebo condition.

In sedentary subjects, salsalate treatment reduced whole cell endothelial NF-κB p65 expression by 24% (P=0.05, Figure 2A) and nuclear endothelial NF-κB p65 expression by 19% (P=0.06, Figure 2B) compared with the placebo condition. Salsalate treatment did not affect whole cell or nuclear endothelial expression of NF-κB p65 in aerobic exercise-trained subjects (P>0.05, Figure 2). Thus, salsalate effectively reduced NF-κB signaling in endothelial cells in subjects with elevated NF-κB at baseline.

NF-κB mediated suppression of EDD. Salsalate treatment resulted in significantly greater improvements in brachial FMD in the sedentary group vs. the exercise-trained group (%FMD: +2.94% vs. +0.4%, absolute FMD: +0.11mm vs. +0.02 mm, respectively, P<0.001). This represents a 76% improvement in percent brachial FMD in the sedentary adults (P<0.01), while there was no improvement in the exercise-trained adults (P>0.05, Figure 3). Similar group differences are present when FMD is expressed as absolute change in diameter (Table 3).

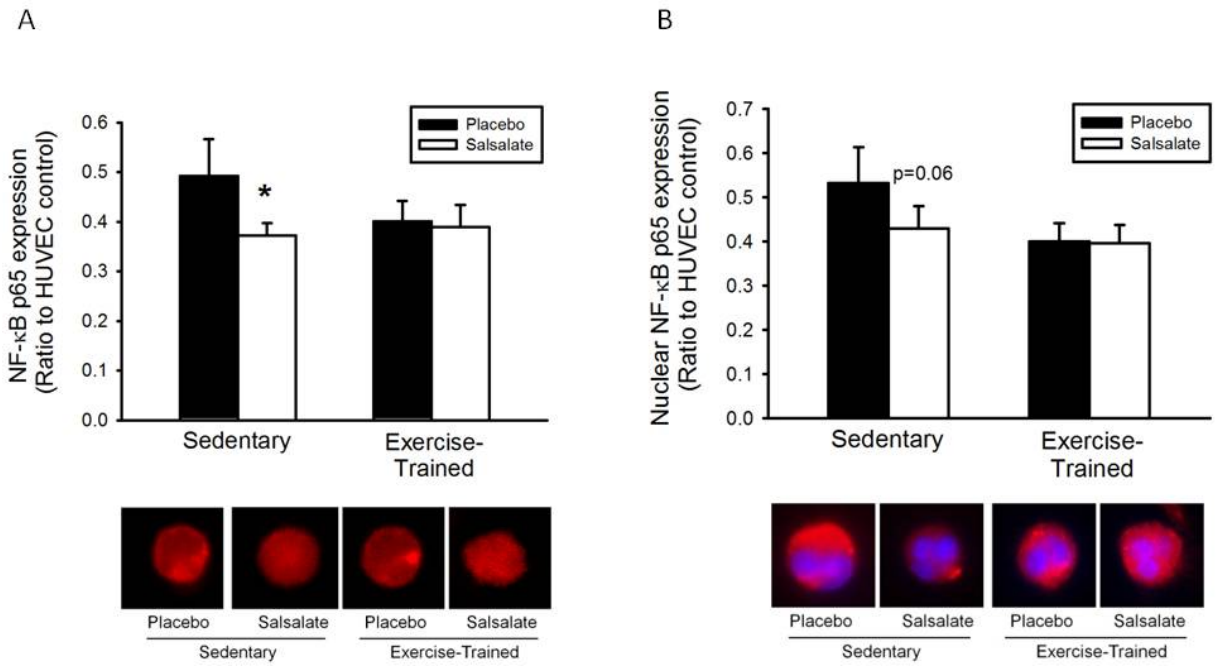

Figure 2. Whole cell (A) and nuclear (B) endothelial protein expression of nuclear factor-κB (NF-κB) p65 in middle-aged and older sedentary and aerobic exercise-trained adults during placebo and salsalate treatment. Representative images are below summary graphs. HUVEC: human umbilical vein endothelial cell. *P<0.05 vs. placebo condition. Values are mean ± SE.

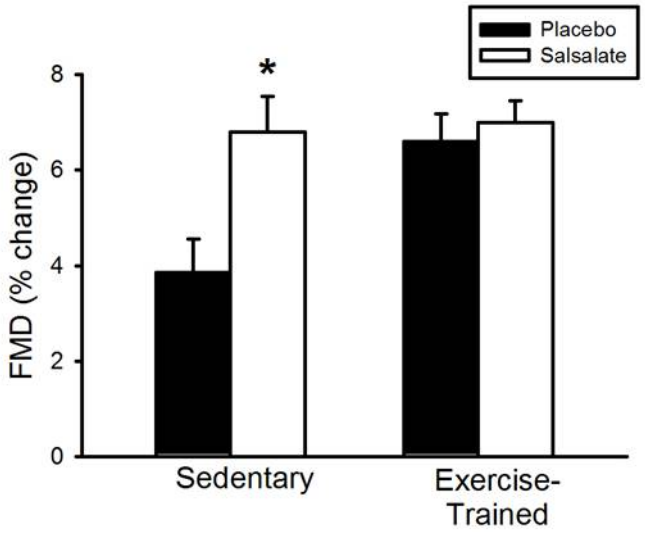

Figure 3. Percent brachial flow-mediated dilation (FMD) in sedentary and aerobic exercise-trained middle-aged and older adults during placebo and salsalate treatment. *P<0.05 vs. placebo condition. Values are mean ± SE.

Table 3: Brachial artery parameters during salsalate and placebo

	Sedentary		Exercise-Trained	
	Placebo	Salsalate	Placebo	Salsalate
Brachial artery diameter (mm)	3.9 ± 0.2	3.9 ± 0.2	3.8 ± 0.1	3.9 ± 0.2
FMD (mm change)	0.14 ± 0.02	0.26 ± 0.02 *	0.24 ± 0.02	0.27 ± 0.01
Peak shear rate (1/s)	837 ± 69	911 ± 85	901 ± 57	876 ± 66
GTN dilation (% change)	21 ± 1	22 ± 2	25 ± 2	23 ± 1
GTN dilation (mm change)	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1

Data are mean ± SE. FMD, flow-mediated dilation; GTN, glyceryl trinitrate. * $P<0.05$ vs. placebo

Baseline brachial artery diameter, peak shear rate and endothelium-independent dilation did not change with salsalate administration in either group (P>0.05, Table 3). These results indicate that NF-κB signaling suppresses EDD in sedentary middle-aged and older adults, but their peers who habitually perform aerobic exercise have an absence of NF-κB -mediated suppression of EDD.

Role of oxidative stress. To examine if NF-κB signaling increases vascular oxidative stress, nitrotyrosine, a marker of oxidative damage, was measured in endothelial cells. Salsalate treatment reduced endothelial expression of nitrotyrosine by 18% in sedentary subjects (P=0.04), but had no effect in aerobic exercise-trained subjects (P=0.39, Figure 4A). Thus, NF-κB signaling increased endothelial oxidative stress in sedentary middle-aged and older adults, whereas aerobic exercise-trained middle-aged and older adults have an absence of NF-κB-mediated increased oxidative stress.

To determine if this NF-κB-mediated increased oxidative stress suppresses EDD, antioxidant vitamin C was infused intravenously during salsalate and placebo conditions. During the placebo condition, vitamin C infusion improved the percent change in brachial FMD by 32% in the sedentary subjects (P=0.001). However, during salsalate treatment, vitamin C infusion did not affect FMD in sedentary subjects (P=0.94). In aerobic-exercise trained subjects, vitamin C infusion did not change FMD during either treatment condition (P>0.05; Figure 4B). Similar group differences are present when FMD is expressed as absolute change in diameter (data not shown). These results indicate that endothelial oxidative stress is a mechanism by which increased NF-κB signaling suppresses EDD in sedentary middle-aged and older adults. The absence of tonically increased NF-κB signaling in middle-aged and older adults who habitually exercise aerobically is associated with an absence of oxidative stress-mediated suppression of EDD.

To explore the mechanisms for the NF-κB-mediated increased oxidative stress, we measured endothelial cell protein expression of NADPH oxidase, a key reactive oxygen species

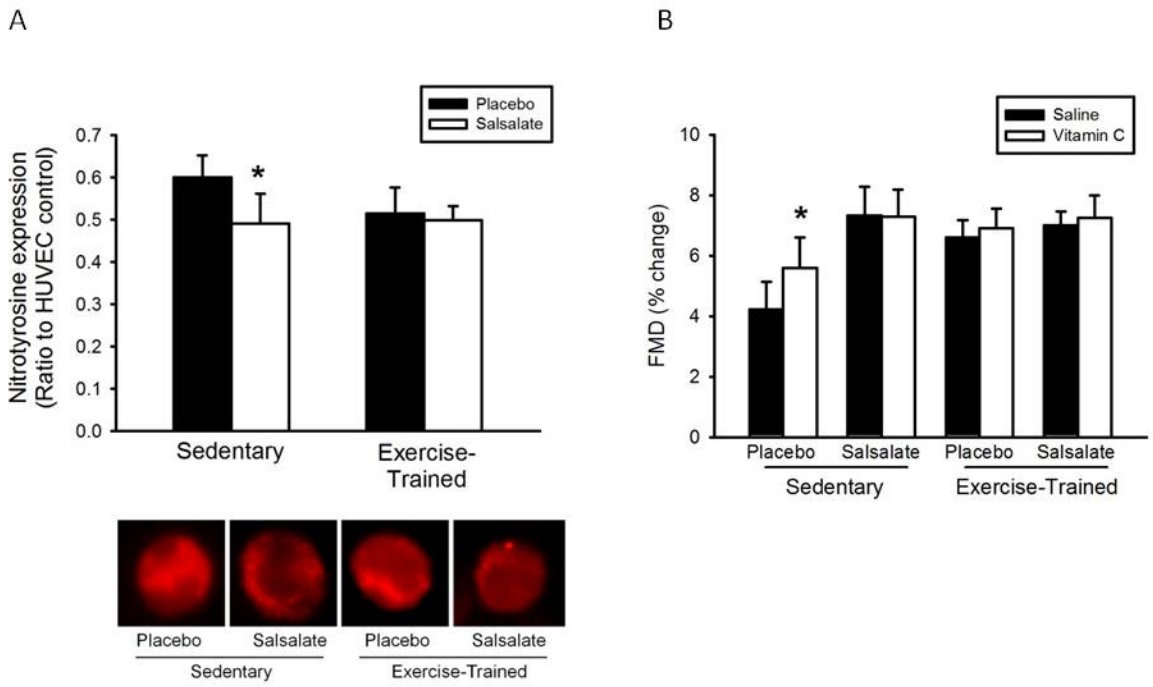

Figure 4. (A) Endothelial cell nitrotyrosine expression in middle-aged and older sedentary and aerobic exercise-trained adults during placebo and salsalate conditions. Representative images are below summary graphs. HUVEC: human umbilical vein endothelial cell. *P<0.05 vs. placebo condition. (B) Percent brachial flow-mediated dilation (FMD) in middle-aged and older sedentary and aerobic exercise-trained adults with intravenous saline or antioxidant vitamin C infusion during placebo and salsalate conditions. *P<0.05 vs. saline infusion. Values are mean ± SE.

producing enzyme in endothelial cells. Salsalate treatment reduced endothelial expression of NADPH oxidase subunit p47 by 30% in sedentary subjects ($P<0.001$), but had no effect in aerobic exercise-trained subjects ($P=0.23$, Figure 5). Thus, increased NADPH oxidase expression is a mechanism by which NF-κB could stimulate oxidative stress in sedentary middle-aged and older adults and this influence is absent in exercise-trained middle-aged and older adults.

Blood pressure, circulating factors and control for potential confounds. Salsalate treatment did not affect diastolic blood pressure, HDL-cholesterol, CRP, IL-6 or TNF-α in either subject group ($P>0.05$). Salsalate treatment reduced total cholesterol, triglycerides and glucose in both sedentary and aerobic exercise-trained subjects ($P<0.05$), while systolic blood pressure and LDL-cholesterol were only reduced in sedentary subjects ($P<0.05$; Table 4). However, changes in these factors did not influence the improvement in FMD with salsalate ($P=0.22$ to 0.60). In addition, group differences in subject characteristics at baseline did not influence the improvement in FMD with salsalate ($P=0.57$ to 0.61).

Discussion

In the present study, we found that aerobic exercise-trained adults are protected against age-related activation of NF-κB and the resulting vascular endothelial dysfunction. In contrast to their sedentary peers, exercise-trained middle-aged and older adults do not demonstrate tonic suppression of EDD by NF-κB signaling. The suppression of EDD by NF-κB signaling in sedentary adults is associated with oxidative stress-mediated impairment of EDD and increased endothelial NADPH oxidase expression. Taken together, these results indicate that middle-aged and older adults who habitually perform aerobic exercise have preserved EDD as a result of less pro-inflammatory signaling and associated oxidative stress.

These findings add to the existing knowledge of the association between inflammatory signaling and EDD. Sedentary aging is thought to be a state of chronic low-grade inflammation,

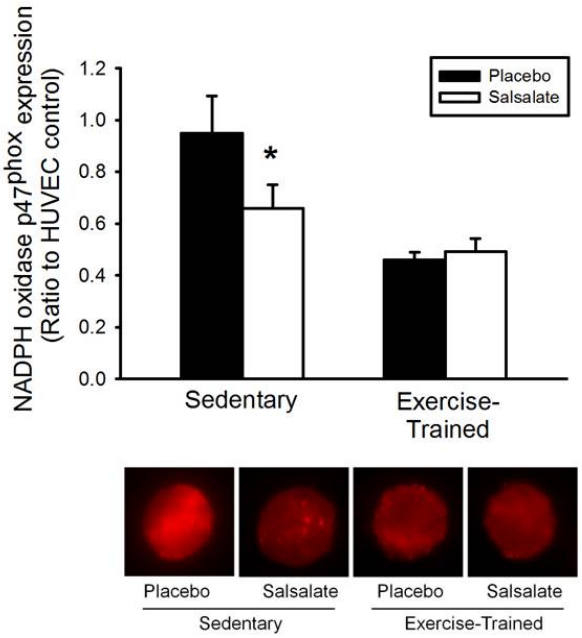

Figure 5. Endothelial cell NADPH oxidase p47 expression in middle-aged and older sedentary and aerobic exercise-trained adults during placebo and salsalate conditions. Representative images are shown below summary graphs. HUVEC: human umbilical vein endothelial cell. . *P<0.05 vs. placebo condition. Values are mean ± SE.

Table 4. Blood pressure and circulating factors

	Sedentary		Exercise-Trained	
	Placebo	Salsalate	Placebo	Salsalate
SBP (mm Hg)	124 ± 5	118 ± 4 *	113 ± 4	111 ± 3
DBP (mm Hg)	73 ± 3	71 ± 3	70 ± 2	68 ± 2
Total cholesterol (mg/dL)	182 ± 8	163 ± 6 *	198 ± 6	172 ± 9 *
LDL-cholesterol (mg/dL)	115 ± 5	103 ± 3 *	125 ± 9	111 ± 9
HDL-cholesterol (mg/dL)	47 ± 3	47 ± 3	51 ± 5	47 ± 4
Triglycerides (mg/dL)	106 ± 14	61 ± 4 *	111 ± 13	69 ± 6 *
Glucose (mg/dL)	89 ± 2	83 ± 2 *	85 ± 1	80 ± 2 *
C-reactive protein (mg/L)	1.2 ± 0.2	1.3 ± 0.2	0.9 ± 0.3	0.8 ± 0.2
Interleukin-6 (pg/mL)	1.9 ± 0.3	1.5 ± 0.2	1.2 ± 0.4	0.7 ± 0.3
TNF-α (pg/mL)	1.2 ± 0.2	1.4 ± 0.2	0.6 ± 0.2	0.8 ± 0.2

Data are mean ± SE. SBP, systolic blood pressure; DBP, diastolic blood pressure; TNF-α, tumor necrosis factor- α. * $P<0.05$ vs. placebo

indicated by increased circulating pro-inflammatory cytokines (4, 15). In addition, pro-inflammatory cytokines and acute phase proteins are positively related to impaired EDD (25, 26). We have previously shown that inhibition of NF-κB signaling improves EDD in overweight and obese adults with characteristics of the metabolic syndrome, such as elevated blood pressure and lipids (20). In the present study, we show that the same signaling pathway suppresses EDD in healthy sedentary middle-aged and older adults.

Habitual aerobic exercise has many positive benefits for cardiovascular health, including a prevention or lessening of age-related declines in vascular function, such as EDD (11-13, 22). Reduced inflammatory signaling has been implicated in this association, as demonstrated by lower circulating CRP, TNF-α and IL-6 in older adults with greater physical activity (6, 24). Here we show a lack of age-related increase in NF-κB expression and activity in individuals who perform habitual aerobic exercise. Administration of salsalate allowed us to mechanistically link the observed findings of lower inflammatory signaling and preserved EDD in these aerobically active adults.

We have previously shown that oxidative stress tonically suppresses EDD in sedentary, but not aerobic exercise-trained middle-aged and older adults (12). In mice, superoxide produced by NADPH oxidase is responsible for this effect, as inhibiting NADPH oxidase with apocynin improves carotid artery EDD in older control mice, but not older mice given access to voluntary wheel running (10). Here, we have shown that NF-κB signaling is a mechanism for the lack of tonic oxidative stress-mediated suppression of EDD in exercise-trained middle-aged and older adults and this is related to a reduced expression of NADPH oxidase in endothelial cells. Thus, we have identified NF-κB signaling as an upstream modulator of endothelial oxidative stress, perhaps via increased reactive oxygen species production by NADPH oxidase.

In the present study, circulating markers of inflammation and oxidative stress did not change with salsalate-induced inhibition of NF-κB signaling in sedentary or exercise-trained adults. We found a similar lack of effect of treatment in obese and overweight adults with

characteristics of the metabolic syndrome (20). This observation may indicate that inhibition of inflammatory signaling and oxidative stress is a local as opposed to a systemic effect. However, this may also be a consequence of the large variability in the measurement of circulating factors in humans, as there were trends for reduced IL-6 in both groups. Most importantly, we did show that salsalate produced endothelial specific effects on pro-inflammatory and pro-oxidant proteins.

We used salsalate for this study because we know it effectively reduces NF-κB expression and activity in endothelial cells, while limiting effects that would confound the interpretation of the results (20). Unlike other salicylates, salsalate lacks the acetyl group necessary to directly inhibit cyclooxygenase-mediated production of prostaglandins (2, 27). Salsalate does not affect endothelial protein expression of cyclooxygenase or plasma prostaglandin $F_{1\alpha}$ (20). With salsalate treatment, there were reductions in systolic blood pressure as well as circulating glucose and lipids. Some of these changes were seen in the sedentary group only, whereas other factors changed in both groups. The changes in circulating glucose and lipids occurred despite a standardization of the subject's diet for 3-days before each measurement of EDD, as we observed in our previous study on overweight and obese adults (19). Nevertheless, we found that differences in these factors did not influence the changes in EDD with salsalate.

We have recently shown that not all middle-aged and older women respond to an aerobic exercise intervention (moderate-intensity daily walking) with improved EDD (19). By a cross-sectional comparison, we also found that women who habitually perform aerobic exercise (vigorous aerobic activity most days) are not protected from the age-related reductions in EDD, on average. That is, some middle-aged and older women do respond to aerobic exercise, but a majority of women do not. In contrast, men who perform habitual aerobic exercise have preserved EDD and all men in the above study responded to the aerobic exercise intervention with improved EDD (19). With these results in mind, in the present study we only included

aerobic exercise-trained women who demonstrated preserved EDD (FMD >6%). Furthermore, the average FMD for men and women did not differ within subject groups in the present study (data not shown). Our results indicate that, similar to men, NF-κB signaling does not suppress EDD in aerobic exercise-trained women who have *preserved* EDD.

There are a few limitations of the present study that are important to note. First, this was a cross-sectional comparison of two groups of middle-aged and older adults with vastly different exercise habits. We do not know if aerobic exercise interventions, such as moderate-intensity daily walking, will have the same effect on inflammatory signaling in endothelial cells. Thus, determining if an aerobic exercise intervention can improve EDD in previously sedentary middle-aged and older adults by reducing inflammatory signaling is an important follow-up study. Reductions in circulating inflammatory markers have been identified with aerobic exercise interventions in the elderly (18), but the effect of reduced inflammatory signaling on EDD is unknown. Therefore, it is important to perform an inhibition of inflammatory signaling, such as with salsalate, before and after an exercise intervention to demonstrate a cause-and-effect relation. Second, we were limited in the number of analyses we could perform on the collected endothelial cells. Our technique for human endothelial cell biopsy only collects a few hundred cells and, as a result, we did not have enough cells to thoroughly study inflammatory and oxidative stress pathways. Likewise, because of our limited cell numbers, we could not measure activity of NADPH oxidase, but only the protein expression of this enzyme. Future studies may explore other potential modulators in endothelial cells, such as antioxidant enzyme expression, as mechanisms for reduced tonic oxidative stress associated with reduced inflammatory signaling in the aerobic exercise-trained state.

There are important clinical implications of identifying interventions to improve EDD in sedentary middle-aged and older adults, as this should lead to reduced risk for cardiovascular diseases and events. Lifestyle interventions, such as aerobic exercise, clearly improve vascular endothelial function in many adults with baseline dysfunction. However, not all middle-aged and

older adults can or will participate in aerobic exercise. Thus, identifying the molecular pathways responsible for such improvements are important and future interventions can be designed to target these pathways. In the present study, we have demonstrated that inflammatory signaling is an important factor in the modulation of EDD among middle-aged and older adults in the absence of clinical disease.

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CHAPTER VI

Conclusions

I used two different approaches to examine the modulation of vascular endothelial function by chronic low-grade inflammation in middle-aged and older adults. First, I found that a greater white blood cell (WBC) count is related to impaired endothelium-dependent dilation (EDD) among middle-aged and older adults. Higher WBC count was associated with impaired EDD via decreased vascular smooth muscle responsiveness to nitric oxide as well as reduced tetrahydrobiopterin and nitric oxide bioavailability. Increased myeloperoxidase production by WBCs may be an important mechanism for these effects. Second, I demonstrated that increased inflammatory signaling is a key mechanism for impaired EDD in healthy middle-aged and older compared with young adults. In sedentary middle-aged and older adults, nuclear factor-κB (NF-κB) tonically suppresses EDD by increasing oxidative stress. However, in aerobic exercise-trained middle-aged and older adults, inflammatory signaling does not suppress EDD. Vascular oxidative stress was an important mechanism mediating the differing effects of aging and regular aerobic exercise on inflammatory signaling-associated modulation of EDD.

In summary, chronic low-grade inflammation modulates EDD in healthy sedentary middle-aged and older adults. However, habitual aerobic exercise preserves EDD by limiting the suppression by inflammatory signaling. These studies have important clinical implications as vascular endothelial dysfunction is an important antecedent to clinical CVD and aging is the major risk factor for CVD. Specifically, the results of my experiments identify factors and mechanisms that modulate vascular endothelial function with aging. These findings can be used to identify novel pharmacologic interventions (e.g., NF-κB inhibitors) and the mechanisms underlying the efficacy of life-style behaviors (e.g., aerobic exercise) that improve vascular endothelial function and reduce the risk of CVD.

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