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 to 75 years (n=48) who have a WBC count within the clinically normal range. The peak forearm blood flow response to intrabrachial artery infusion of acetylcholine was inversely related to WBC count \((r=-0.38; P=0.004)\) and was 34% smaller in subjects with higher versus lower WBC count (more versus less than the median of \(5.0\times10^9\) cells per liter; \(P=0.001)\). Vascular smooth muscle responsiveness to NO (peak forearm blood flow 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\(^{G\text{-}}\)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 with a 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. (Hypertension. 2010;55:363-369.)

Key Words: endothelium-dependent dilation ■ aging ■ NO ■ tetrahydrobiopterin ■ neutrophils
NO and produces reactive oxygen species that oxidize tetrahydrobiopterin, collectively resulting in reduced NO bioavailability.\textsuperscript{14,15} Higher circulating concentrations of myeloperoxidase are associated with impaired EDD in patients with rheumatoid arthritis\textsuperscript{16} and cardiovascular disorders,\textsuperscript{17} 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 nonsmoking, 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 to 75 years. We then determined whether EDD differed in groups of middle-aged and older adults with lower versus higher WBC count compared with 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 to 75 years. The subjects were divided into 2 equal groups on the basis of the median WBC count (5.0×10\textsuperscript{9} cells per liter). Reference data for EDD and NO responsiveness were included on a group of healthy young adult controls (18 to 35 years; n=17; 13 men and 4 women). Subjects were free of clinical CVD, diabetes mellitus, 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 before the study. No subjects had an abnormally high WBC count (>10.0×10\textsuperscript{9} cells per liter) that would indicate an acute inflammatory response. All of the 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 before participation.

Procedures
All of the measurements were performed at the University of Colorado at Boulder Clinical and Translational Research Center after a 12-hour fast and a 24-hour abstinence 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). Fasting plasma metabolic factors were determined by the Clinical and Translational Research Center core laboratory using standard assays. WBC count was measured by standard Coulter counter technique (Beckman Coulter Ac T Sdif CFP, ELISA was used to measure serum concentrations of myeloperoxidase (Prognostix), oxidized low-density lipoprotein (LDL; ALPCO), tumor necrosis factor-\(\alpha\), and interleukin (IL)-6 (R&D Systems). C-reactive protein was measured using a high-sensitivity Chemistry Immuno Analyzer (AU400e, Olympus America).

Vasodilatory Responses
Forearm blood flow (FBF) responses to incremental intrabrachial artery infusion of acetylcholine (1.0, 2.0, 4.0, and 8.0 \(\mu\)g per 100 mL, forearm volume per minute; ie, EDD) and sodium nitroprusside (0.5, 1.0, and 2.0 \(\mu\)g·min\(^{-1}\)·100 mL per forearm volume per minute) were measured in the experimental (nondominant) and the control (dominant) forearm of all of the subjects using strain-gauge venous occlusion plethysmography (Hokanson), as described previously.\textsuperscript{18–20} The contribution of NO to the FBF responses to acetylcholine was determined in a subset of subjects (lower WBC count: n=4, 2 men and 2 women; higher WBC count: n=4, 3 men and 1 woman) by coinfusion of N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA; Clinalfa; 5 mg/min; 10-minute 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, 4 men and 7 women; higher WBC count: n=12, 5 men and 7 women) by coinfusion of tetrahydrobiopterin (Clinalfa; 500 \(\mu\)g/min; 10-minute 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 effects 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 sodium nitroprusside and acetylcholine (alone and during coinfusion of tetrahydrobiopterin and/or L-NMMA) 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 of the 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lower WBC Count</th>
<th>Higher WBC Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (men/women)</td>
<td>24 (13/11)</td>
<td>24 (13/11)</td>
</tr>
<tr>
<td>WBC count, 10\textsuperscript{9} cells per L</td>
<td>4.1±0.1</td>
<td>6.0±0.2*</td>
</tr>
<tr>
<td>Age, y</td>
<td>63±1</td>
<td>63±1</td>
</tr>
<tr>
<td>Body mass index, kg/m\textsuperscript{2}</td>
<td>26±1</td>
<td>28±1</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.85±0.02</td>
<td>0.86±0.02</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>123±3</td>
<td>121±2</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>75±2</td>
<td>75±2</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>210±5</td>
<td>198±6</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>117±5</td>
<td>115±5</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>60±3</td>
<td>53±3</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>110±10</td>
<td>121±8</td>
</tr>
<tr>
<td>Fasting blood glucose, mg/dL</td>
<td>88±1</td>
<td>95±2*</td>
</tr>
<tr>
<td>C-Reactive protein, mg/L</td>
<td>1.0±0.3</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>1.2±0.2</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>Tumor necrosis factor-(\alpha), pg/mL</td>
<td>1.5±0.2</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>Myeloperoxidase, pmol/L</td>
<td>327±30</td>
<td>465±52*</td>
</tr>
<tr>
<td>Oxidized LDL, U/L</td>
<td>60±3</td>
<td>59±3</td>
</tr>
</tbody>
</table>

Data are mean\(±\)SE unless otherwise specified. HDL indicates high-density lipoprotein.

*\(P<0.05\) vs lower WBC count.
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, tumor necrosis factor-α, and oxidized LDL were not different in the 2 groups.

Vasodilatory Responses

EDD

Among all of the 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 on the basis of 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 counts were smaller (P=0.006) and not different (P=0.55), respectively, compared with young adult controls (age: 25±1 years; WBC count: 5.4×10⁹ cells per liter). 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 of the 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 are reduced in middle-aged and older adults with a higher WBC count.

To determine whether 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 sensitivity to NO contributes to, but does not completely explain, the greater impairments in EDD in the subjects with a higher compared with a 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 acetylcholine in both groups (both P≤0.008; Figure 3), abolishing the vasodilatory-enhancing effects of tetrahydrobiopterin in the subjects with a higher WBC count. These findings provide evidence that reduced NO bioavailability was the mechanism by which reduced tetrahydrobiopterin bioactivity impaired FBF responses to acetylcholine in subjects with higher WBC count.
Among all of the subjects, neutrophil count demonstrated the strongest relation to the peak FBF response to acetylcholine ($r = 0.38; P = 0.005; \text{Figure 4A}$). Eosinophil and monocyte counts also were related to the peak FBF response to acetylcholine (Table 2). Basophil and lymphocyte counts 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.

**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 ($r = -0.38; P = 0.005; \text{Figure 4A}$). Eosinophil and monocyte counts also were related to the peak FBF response to acetylcholine (Table 2). Basophil and lymphocyte counts 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.

**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, tumor necrosis factor-$\alpha$, or oxidized LDL. Collectively, these results indicate that WBC count was the best predictor of EDD among all of the 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 nonsmoking, 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 (r = 0.32; P = 0.02), and fasting blood glucose (r = 0.45; P = 0.001).

Table 2. Associations Between Types of WBCs and EDD

<table>
<thead>
<tr>
<th>WBC Differential Count, 10^9 Cells per L</th>
<th>Peak FBF Response to ACh, mL per 100 mL of FAV per Minute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>-0.38</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>-0.30</td>
</tr>
<tr>
<td>Monocyte</td>
<td>-0.27</td>
</tr>
<tr>
<td>Basophil</td>
<td>-0.20</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>-0.15</td>
</tr>
</tbody>
</table>

ACh indicates acetylcholine; FAV, forearm volume.

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 mellitus.11 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 cGMP signaling.21 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 were 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 cofactor for NO synthase. Infusion of tetrahydrobiopterin improves EDD on average in middle-aged and older adults,12 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 nonsmoking 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,22 with a consequent reduction in NO bioavailability. Consistent with this idea, we found that coinfusion 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 2 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 nitroprusside 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 a result of 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 significant predictor of future cardiovascular events. 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.

Myeloperoxidase
Because neutrophils produce the majority of circulating myeloperoxidase, and serum myeloperoxidase is a predictor of EDD in patients with clinical disease, 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. 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 with a moderately increased risk of CVD. In contrast, serum concentrations of the cytokines IL-6 and tumor necrosis factor-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 the 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. On interacting with the vascular wall, WBCs can produce and release reactive oxygen species and cytokines, which could, in turn, influence gene and protein expression, intracellular signaling, and vasodilatory responsiveness. 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 nonsmoking 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 with 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 a result of the 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 cardiovascular events.

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Disclosures
None.

References


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