Altered Plasma Versus Vascular Biopterins in Human Atherosclerosis Reveal Relationships Between Endothelial Nitric Oxide Synthase Coupling, Endothelial Function, and Inflammation

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Background—Tetrahydrobiopterin (BH₄) is a key regulator of endothelial nitric oxide synthase (eNOS) activity and coupling. However, the extent to which vascular and/or systemic BH₄ levels are altered in human atherosclerosis and the importance of BH₄ bioavailability in determining endothelial function and oxidative stress remain unclear. We sought to define the relationships between plasma and vascular biopterin levels in patients with coronary artery disease and to determine how BH₄ levels affect endothelial function, eNOS coupling, and vascular superoxide production.

Methods and Results—Samples of saphenous veins and internal mammary arteries were collected from 219 patients with coronary artery disease undergoing coronary artery bypass grafting. We determined plasma and vascular levels of biopterins, vasomotor responses to acetylcholine, and vascular superoxide production in the presence and absence of the eNOS inhibitor N⁵-nitro-L-arginine methyl ester. High vascular BH₄ was associated with greater vasorelaxations to acetylcholine (P<0.05), whereas high plasma BH₄ was associated with lower vasorelaxations in response to acetylcholine (P<0.05). Furthermore, an inverse association was observed between plasma and vascular biopterins (P<0.05 for both saphenous veins and internal mammary arteries). High vascular (but not plasma) BH₄ was associated with reduced total and N⁵-nitro-L-arginine methyl ester–inhibitable superoxide, suggesting improved eNOS coupling. Finally, plasma but not vascular biopterin levels were correlated with plasma C-reactive protein levels (P<0.001).

Conclusions—An inverse association exists between plasma and vascular biopterins in patients with coronary artery disease. Vascular but not plasma BH₄ is an important determinant of eNOS coupling, endothelium-dependent vasodilation, and superoxide production in human vessels, whereas plasma biopterins are a marker of systemic inflammation. (Circulation. 2007;116:2851-2859.)

Key Words: atherosclerosis ■ endothelium ■ free radicals ■ nitric oxide ■ nitric oxide synthase

Tetrahydrobiopterin (BH₄) is an essential cofactor for endothelial nitric oxide synthase (eNOS) and is required for nitric oxide (NO) synthesis in the vascular endothelium. Reduced BH₄ availability leads to eNOS uncoupling and the production of superoxide radicals instead of NO. Recent evidence suggests that BH₄-dependent eNOS uncoupling may be an important mechanism that mediates endothelial dysfunction and increased superoxide generation in vascular disease states.

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Several studies have found that BH₄ levels in either endothelial cells or vascular tissues are reduced in vascular disease models. However, BH₄ biosynthesis can be increased by cytokine stimulation in inflammatory cells and in cultured endothelial cells through induction of the rate-limiting enzyme GTP cyclohydrolase I (GTPCH). Because both local and systemic inflammation are features of atherosclero-

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sis, it is unclear how vascular disease states could be associated with reduced biopterin biosynthesis as the sole cause of BH4 deficiency. Indeed, plasma levels of neopterin, a metabolic by-product of biopterin biosynthesis, are elevated in patients with inflammatory conditions, including coronary artery disease (CAD).11,12 In experimental models of vascular disease, some studies have reported a reduction in GTPCH levels, leading to reduced biopterin synthesis.13 Other studies have suggested that reduced vascular BH4 is due to oxidation of BH4 by reactive oxygen species such as peroxynitrite11,14 to form dihydrobiopterin (BH2) and finally biopterin.11,15 Loss of BH4 through oxidation adds additional mechanistic complexity because selective changes in BH4 could be masked by little or no change in the overall level of total biopterins, comprising the sum of BH4, BH2, and biopterin. Furthermore, no studies have examined whether vascular biopterins are regulated in parallel with systemic biopterin levels. Indeed, the extent to which vascular and/or systemic biopterin levels are altered in human atherosclerosis and the importance of BH4 levels in determining endothelial function remain unclear.

Accordingly, we sought to systematically define the relationships between plasma and vascular biopterin levels in patients with CAD and to determine how BH4 levels influence eNOS coupling, endothelial function, and inflammation in human atherosclerosis.

Methods

Study Subjects
For the present study we initially screened 303 patients with CAD undergoing elective coronary bypass grafting (CABG) at the John Radcliffe Hospital, Oxford, UK. Of these subjects, 219 fulfilled the inclusion criteria and agreed to participate. Exclusion criteria were any inflammatory, infective, liver, or renal disease, overt clinical heart failure, malignancy, or acute coronary event during the last 2 months. Patients receiving nonsteroidal anti-inflammatory drugs, dietary supplements of folic acid, or antioxidant vitamins were also excluded. Individual characteristics of the patients are presented in Table 1. The study was approved by the local Research Ethics Committee, and each patient gave written informed consent.

Tissue and Plasma Samples
Samples of saphenous vein (SV) (n = 101) and internal mammary artery (IMA) (n = 101) were obtained at the time of CABG, as we have described previously.16 Paired vessel segments were snap frozen and stored at −80°C for measurement of biopterin content or were transferred to the laboratory for functional studies within 30 minutes in ice-cold Krebs-Henseleit buffer. For endothelial denudation experiments, endothelium was removed by gently pulling the vessel ring over a 2-mm-diameter wooden stick to produce mild abrasion of the luminal surface of the vessel, without undue stretching, followed by a flushing of the lumen with PBS to remove any residual endothelial debris, as described previously.16 Because the number of assays performed on each vessel was limited by the small quantity of tissue, a subset of the total population was studied in each individual experiment. Blood samples were obtained immediately before surgery, after overnight fasting. Samples were centrifuged at 2500 rpm for 10 minutes, and serum/plasma was stored at −80°C.

Oxidative Fluorescent Microphotography
In situ superoxide production was determined in vessel cryosections with the oxidative fluorescent dye dihydroethidium.16,17 Cryosections (30 μm) were incubated with dihydroethidium (2 μmol/L) in Krebs-HEPES buffer, with or without Nω-nitro-L-arginine methyl ester (L-NAME) (100 μmol/L).

Table 1. Individual Characteristics of Patients

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>219</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men/women</td>
<td>188/31</td>
</tr>
<tr>
<td>Age, y</td>
<td>65.91 ± 8.9</td>
</tr>
<tr>
<td>SV, n</td>
<td>101</td>
</tr>
<tr>
<td>IMA, n</td>
<td>101</td>
</tr>
<tr>
<td>Risk factors, n (%)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>144 (65)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>160 (73)</td>
</tr>
<tr>
<td>Smokers/ex-smokers</td>
<td>29/114 (13/52)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>61 (28)</td>
</tr>
<tr>
<td>Family history</td>
<td>115 (53)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.7 ± 7.5</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.60 ± 0.80</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>3.98 ± 0.88</td>
</tr>
<tr>
<td>High-density lipoprotein, mmol/L</td>
<td>1.09 ± 0.26</td>
</tr>
<tr>
<td>Ejection fraction of left ventricle, %</td>
<td>52.0 ± 11.2</td>
</tr>
<tr>
<td>Plasma biopterin levels</td>
<td></td>
</tr>
<tr>
<td>Plasma BH₄, nmol/L</td>
<td>21.3 (11.5–42.1)</td>
</tr>
<tr>
<td>Plasma BH₂, nmol/L</td>
<td>15.1 (11.6–19.2)</td>
</tr>
<tr>
<td>Plasma biopterin, nmol/L</td>
<td>3.89 (2.57–6.58)</td>
</tr>
<tr>
<td>Plasma total biopterin, nmol/L</td>
<td>46.9 (30.9–70.3)</td>
</tr>
<tr>
<td>BH₄/total biopterin ratio</td>
<td>0.51 ± 0.24</td>
</tr>
<tr>
<td>Angiographic extent of CAD, n (%)</td>
<td></td>
</tr>
<tr>
<td>1-vessel disease</td>
<td>6 (3)</td>
</tr>
<tr>
<td>2-vessel disease</td>
<td>59 (27)</td>
</tr>
<tr>
<td>3-vessel disease</td>
<td>154 (70)</td>
</tr>
<tr>
<td>Medication, n (%)</td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>200 (91)</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitors</td>
<td>137 (63)</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>68 (31)</td>
</tr>
<tr>
<td>Angiotensin receptor blockers</td>
<td>16 (7)</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>181 (83)</td>
</tr>
<tr>
<td>Nitrites</td>
<td>99 (45)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>186 (85)</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>64 (29)</td>
</tr>
<tr>
<td>Diuretics</td>
<td>49 (22)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD or as median (25th to 75th percentile), unless otherwise indicated.

Fluorescent images of the endothelium (×40, Zeiss LSM 510 META laser scanning confocal microscope, Carl Zeiss, Inc, Oberkochen, Germany) were obtained from each vessel quadrant. In each case, segments of vessel rings (with and without L-NAME) were analyzed in parallel with identical imaging parameters. Dihydroethidium fluorescence was quantified by automated image analysis with Image-Pro Plus software (Media Cybernetics, Bethesda, Md); all analyses were performed in a blinded fashion by 2 independent observers.

Vasomotor Studies
Endothelium-dependent and endothelium-independent dilatations were assessed in SV obtained at the time of CABG with the use of isometric tension studies.16 Four rings from each vessel were precontracted with phenylephrine (3 × 10⁻⁶ mol/L), then endotheli-
um-dependent relaxations were quantified with acetylcholine (10^{-9} to 10^{-5} mol/L). Finally, relaxations to the endothelium-independent NO donor sodium nitroprusside (SNP) (10^{-10} to 10^{-6} mol/L) were evaluated in the presence of the NOS inhibitor L-NAME (100 μmol/L), as we have described previously.16

**Determination of Vascular Superoxide Production**

Vascular superoxide production was measured in paired segments of SV and IMA with the use of lucigenin-enhanced chemiluminescence, as described previously.16,18 Vessels were opened longitudinally to expose the endothelial surface and equilibrated for 20 minutes in oxygenated (95% O_2/5% CO_2) Krebs-HEPES buffer (pH 7.4) at 37°C. Lucigenin-enhanced chemiluminescence was measured with the use of low-concentration lucigenin (5 μmol/L).16 NOS-derived superoxide production was determined by the difference in superoxide production after incubation with the NOS inhibitor L-NAME (100 μmol/L).

**Determination of Plasma and Vascular Biopterin Levels**

BH4, BH3, and biopterin levels in plasma or vessel tissue lysates were each determined separately by high-performance liquid chromatography followed by electrochemical (for BH4) and fluorescent (for BH and biopterin) detection, as described previously,19 with some modifications. Biopterin levels were expressed as pmol/g of tissue for vessels and nmol/L for plasma (for details, see the online-only Data Supplement).

**Western Blotting**

Protein was extracted from frozen segments of SV with lysis buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 1% Nonidet P-40) containing protease inhibitors (Complete; Roche) and 1 mmol/L phenylmethylsulfonyl fluoride. Protein lysates (5 to 15 μg) were separated by electrophoresis on 4% to 12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, Calif). eNOS and GAPDH were detected with the use of mouse monoclonal antibodies (BD Biosciences, San Jose, Calif, and Chemicon International, Temecula, Calif, respectively) and Tie-2 with the use of a goat polyclonal antibody (R&D Systems, Minneapolis, Minn). eNOS/Tie-2 levels were normalized to GAPDH for quantification.

**Determination of Serum C-Reactive Protein Levels**

Serum levels of C-reactive protein (CRP) were measured by immunonephelometry with a high-sensitivity method (Dade Behring Marburg GmbH, Marburg, Germany).

**Statistical Analysis**

All variables were tested for normal distribution with the use of the Kolmogorov-Smirnov test. Non-normally distributed variables were log-transformed for analysis and are presented as median (25th to 75th percentiles) and range. Comparisons of categorical variables between groups were performed by χ² test. Comparisons of continuous variables between 3 groups (ie, between the 3 tertiles of a splitting variable) were performed by 1-way ANOVA followed by Bonferroni post hoc analysis. Comparisons of continuous variables between 2 independent groups (ie, for the L-NAME–induced change in superoxide production between the 2 groups used for the dihydroethidium experiment) were performed by unpaired t test. The dose–response curves for the vasomotor responses to acetylcholine and SNP were compared between groups by ANOVA for repeated measurements with the use of a dose (factor 1) by group (factor 2) interaction. Correlations between continuous variables were assessed by determining the Pearson correlation coefficient.

In linear multiple regression, we used the following as dependent variables in 4 respective models: (1) total vascular superoxide production in SV rings; (2) L-NAME–inhibitable vascular superoxide production in SV rings; (3) plasma BH4; and (4) plasma total biopterin levels (for details, see the online-only Data Supplement). All probability values were 2-tailed, and P<0.05 was considered statistically significant. All statistical analyses were performed with the use of SPSS 12.0.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

**Results**

**Associations Between Vascular and Plasma Biopterins in Patients With CAD**

We first compared levels of total biopterins in plasma and in vascular tissue (IMA [n=67] and SV [n=77]) collected from patients undergoing CAGB. We divided our population into tertiles according to plasma total biopterin levels, and we observed that plasma and vascular levels of total biopterins varied widely between individual patients (Table 2). However, we found an inverse relationship between plasma and vascular total biopterins in both IMA (r = -0.251, P = 0.051) and SV (r = -0.317, P = 0.007). Indeed, the 3-fold increase in plasma total biopterins between the highest and lowest tertiles was associated with a 2-fold reduction in vascular total biopterins (Figure 1). In addition, no significant association was present between plasma and vascular absolute BH4 levels, suggesting that they behave as 2 independent compartments (Figure 1). Measurement of vascular biopterins in paired vascular segments with or without endothelial denudation revealed that 88.8±3.6% of vascular BH4 and 75.0±4.5% of vascular total biopterins were localized to the endothelium (Figure 2). Denudation of the vascular endothelium in these vessels was confirmed by Western blotting of endothelium-specific proteins eNOS and Tie-2 in vascular segments from 4 patients (Figure 2). These findings suggest that vascular biopterin levels are determined principally by endothelial biopterin content and that vascular and plasma biopterin levels are regulated differentially in patients with CAD.

**Vascular and Plasma BH4 Levels and Endothelial Function**

We next examined whether vascular biopterins were associated with relaxation responses of SV (n=74) to acetylcholine as a measure of NO-mediated endothelial function. To address this question, we divided our population into tertiles according to vascular total biopterins or vascular BH4 levels, and we compared the vasomotor responses of SV to acetylcholine between the 3 tertiles. No significant relationship existed between vascular total biopterins and acetylcholine relaxations (P=0.1). However, analysis of acetylcholine dose–response curves according to tertiles of vascular BH4 revealed that patients with vascular BH4 in the highest and middle tertiles had significantly greater relaxations to acetylcholine compared with those in the lowest tertile (Figure 3A). In contrast, no differences were present between vascular BH4 tertiles in relaxation responses to the direct NO donor SNP (Figure 3B). Surprisingly, the relationship between plasma BH4 and relaxation to acetylcholine was the inverse of the association observed between vascular BH4 and acetylcholine relaxations. Patients with plasma BH4 in the highest tertile had significantly reduced NO-mediated endothelial function compared with patients in the lowest tertile (Figure 3C),
patients in the highest tertile of vascular BH4 had significantly lower superoxide production in both SV (n
lower superoxide production in both SV (n
both vascular total superoxide (β [SE]=−2.487 [0.572];
P=0.0001) and L-NAME–induced change in vascular superoxide (β [SE]=2.895 [0.643]; P=0.0001) in SV. To visualize the relationship between vascular BH4 and superoxide production, we performed dihydroethidium staining in SV from 5 patients at the highest and 5 patients at the lowest tertile of vascular BH4. We observed that endothelium-derived superoxide was decreased by L-NAME in vessels with low BH4 (by −4.7±0.2 U/mm of endothelium), suggesting eNOS uncoupling, whereas the opposite was observed in vessels with high BH4 levels (by +3.7±2.9 U/mm of endothelium; 
P<0.05 versus low BH4) (Figure 5). Taken together, these findings suggest that vascular BH4 may be a key regulator of eNOS coupling in the vasculature of patients with CAD.

We next examined possible relationships between plasma BH4 and vascular superoxide production. We observed that plasma BH4 was not associated with either total vascular superoxide or the L-NAME–induced change in vascular superoxide in either SV or IMA (Figure 6A through 6D). Correspondingly, no association existed between plasma BH4 and the ratio BH4/(BH4+biopterin) in plasma (Figure 6E and 6F). These findings suggest that vascular rather than plasma BH4 has a greater impact on eNOS coupling and vascular superoxide production. To further evaluate the biological importance of eNOS coupling, as determined by L-NAME–inhibitable vascular superoxide production, we examined whether this measure was related to NO-mediated endothelial function. Indeed, L-NAME–induced change of vascular su-

Vascular and Plasma BH4 Levels and Superoxide Production in Human SV and IMA
To further examine the biological significance of plasma and vascular biopterins in human atherosclerosis, we examined the relationship between vascular BH4 and vascular superoxide production. We divided our population into tertiles according to vascular BH4 levels, and we observed that patients in the highest tertile of vascular BH4 had significantly lower superoxide production in both SV (n=90) and IMA (n=82) than those in the lowest vascular BH4 tertile (Figure 4A and 4B). Because BH4 regulates eNOS coupling, we then examined whether vascular BH4 levels may affect eNOS-dependent superoxide production by measuring the change in vascular superoxide induced by eNOS inhibition. Indeed, we observed that the L-NAME–induced decrease in vascular superoxide was significantly greater in SV and IMA from patients with low vascular BH4 compared with those with high vascular BH4, reflecting greater eNOS coupling in the presence of high vascular BH4 (Figure 4C and 4D). In addition, a significant association was present between vascular BH4 and the ratio of BH4/(BH4+biopterin) in both vessel types (Figure 4E and 4F), suggesting that higher absolute levels of vascular BH4 are associated with less BH4 oxidation. On the other hand, no association existed between vascular BH4 (or biopterin) and vascular superoxide generation (data not shown). In multivariate analysis, we found that vascular BH4 was the only independent predictor of both vascular total superoxide (β [SE]=−2.487 [0.572]; 
P<0.001) and L-NAME–induced change in vascular superoxide (β [SE]=2.895 [0.643]; 
P=0.0001) in SV. To visualize the relationship between vascular BH4 and superoxide production, we performed dihydroethidium staining in SV from 5 patients at the highest and 5 patients at the lowest tertile of vascular BH4. We observed that endothelium-derived superoxide was decreased by L-NAME in vessels with low BH4 (by −4.7±0.2 U/mm of endothelium), suggesting eNOS uncoupling, whereas the opposite was observed in vessels with high BH4 levels (by +3.7±2.9 U/mm of endothelium; 
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whereas no significant association existed between plasma total biopterins and vasomotor responses to acetylcholine (P=0.647). No significant association existed between the absolute values of vascular BH4 or biopterin or the vasomotor responses to acetylcholine (P=NS for all). Relaxation responses to SNP were also not significantly different between plasma BH4 tertiles (Figure 3D).

Table 2. Relationship Between Plasma and Vascular Biopterins, Stratified According to Plasma Total Biopterin Levels

<table>
<thead>
<tr>
<th>Plasma Total Biopterins</th>
<th>Tertile 1 (Low)</th>
<th>Tertile 2 (Mid)</th>
<th>Tertile 3 (High)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH4‡</td>
<td>9.85 (4.04–14.54)</td>
<td>26.29 (19.00–32.75)</td>
<td>54.81 (34.21–70.80)‡‡</td>
</tr>
<tr>
<td>Biopterin†</td>
<td>3.24 (2.33–4.29)</td>
<td>4.14 (2.56–6.41)</td>
<td>5.21 (2.77–9.27)†</td>
</tr>
<tr>
<td>SV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total biopterins‡</td>
<td>71.5 (39.1–239.2)</td>
<td>35.0 (20.2–58.3)</td>
<td>32.7 (22.2–52.4)</td>
</tr>
<tr>
<td>BH4‡</td>
<td>8.9 (5.6–17.2)</td>
<td>8.5 (4.6–12.2)</td>
<td>9.1 (5.3–15.1)</td>
</tr>
<tr>
<td>BH4*</td>
<td>46.6 (24.8–135.9)</td>
<td>23.3 (8.2–39.8)§</td>
<td>19.2 (9.4–45.9)§</td>
</tr>
<tr>
<td>Biopterin*</td>
<td>6.3 (2.2–69.6)</td>
<td>2.3 (2.2–5.9)§</td>
<td>2.3 (2.1–4.5)§</td>
</tr>
<tr>
<td>IMA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total biopterins*</td>
<td>55.7 (26.9–164.3)</td>
<td>31.1 (14.1–79.6)§</td>
<td>29.6 (11.5–61.1)§</td>
</tr>
<tr>
<td>BH4*</td>
<td>10.9 (6.02–18.9)</td>
<td>6.4 (3.2–13.5)§</td>
<td>5.7 (2.7–14.6)§</td>
</tr>
<tr>
<td>BH4*</td>
<td>33.6 (14.5–114.1)</td>
<td>21.4 (8.4–39.3)§</td>
<td>16.9 (7.1–48.7)§</td>
</tr>
<tr>
<td>Biopterin*</td>
<td>10.4 (2.2–48.9)</td>
<td>2.3 (2.2–7.6)§</td>
<td>2.3 (2.2–7.0)§</td>
</tr>
</tbody>
</table>

Values are shown as median (25th to 75th percentile), expressed as nmol/L for plasma biopterins and pmol/g tissue for vascular biopterins. No significant differences existed in individual characteristics of the patients between the 3 groups.

*P<0.05, †P<0.01, ‡P<0.001 derived from 1-way ANOVA across the 3 groups; §P<0.05, ¶P<0.01 vs tertile 1; #P<0.05, **P<0.001 vs tertile 2 after Bonferroni post hoc analysis.
Biopterin Biosynthesis

Because GTPCH activity is induced by proinflammatory stimulation, we examined whether inflammation could explain the discordance between plasma and vascular biopterins. We considered total biopterins (the sum of BH4, BH2, and biopterin) as an index of the overall activity of the biopterin biosynthetic pathway. Indeed, serum CRP was significantly correlated with both plasma total biopterins (Figure 8) and, less strongly, with plasma BH4 (r=0.207, P=0.005) but not with either vascular total biopterins (Figure 8) or vascular BH4 (IMA: r=0.049, P=0.620; SV: r=-0.097, P=0.313). To search for other determinants of plasma and vascular total biopterins, we performed a multivariate analysis, in which we included as independent variables all the demographic characteristics and CRP levels. We observed that plasma CRP was the only independent predictor of both plasma total biopterins (β [SE]=0.103 [0.041]; P=0.012) and plasma BH4 (β [SE]=0.202 [0.184]; P=0.013), suggesting that inflammation is a major determinant of plasma biopterin levels.

Discussion

In the present study we examined the relationships between plasma and vascular biopterin levels, endothelial function, and vascular superoxide production in patients with CAD. First, we observed that plasma biopterin levels are inversely associated with vascular biopterin levels in both arteries and veins. Second, the overwhelming majority of vascular biopterins are present in the endothelium. Third, vascular BH4 levels are inversely associated with vascular superoxide production and positively associated with eNOS coupling and NO-mediated endothelial function in both arteries and veins, independently of the patient’s individual characteristics. Finally, plasma BH4 is positively correlated with CRP levels and inversely associated with endothelial function. These findings suggest a distinct and contrasting biological importance for systemic versus plasma biopterins in patients with CAD. Vascular biopterins are associated with maintained eNOS coupling and endothelial function, whereas plasma biopterins are associated with inflammation and impaired endothelial function.

The proposed importance of BH4 in vascular homeostasis relates to its role as an essential cofactor for eNOS. Recent studies have shown that BH4 availability mediates coupling of oxygen reduction to heme-catalyzed l-arginine oxidation to form NO and l-citrulline. Therefore, BH4 deficiency is believed to lead to eNOS uncoupling, resulting in impaired endothelium-dependent vasodilation and the production of superoxide radicals from the uncoupled enzymatic form. In experimental models, risk factors for atherosclerosis are accompanied by endothelial dysfunction.

Figure 2. Contribution of endothelium to vascular biopterin levels in human vessels. Paired rings of SV from 4 patients were analyzed either intact or after mechanical removal of endothelial layer. Left, Western blotting for endothelium-specific markers such as eNOS and Tie-2 proteins, but not GAPDH, was greatly diminished in denuded rings, confirming endothelial removal. Right, Vascular total biopterins and BH4 were significantly reduced in denuded vessels (by 88.8±3.6% and 75.0±4.5%, respectively). Values are expressed as mean±SEM. *P<0.01 vs without endothelium removal.
However, the role of vascular BH₄ in endothelial function and eNOS coupling in human vessels has not been examined previously. We now demonstrate that vascular BH₄ levels are associated with endothelium-dependent vasodilatation in patients with CAD, a finding supportive of our previous observation that folate-induced elevation of vascular BH₄ leads to an improvement of endothelium-dependent vasodilatation in human vessels.¹⁶ We found corresponding associations between vascular BH₄, vascular superoxide production, and eNOS coupling. Furthermore, absolute vascular BH₄ is associated with the ratio of BH₄/(BH₂+biopterin), a marker of BH₄ oxidation. Despite the inclusion of BH₄ in the mathematical numerator of this ratio, a dynamic conversion exists of BH₄ to BH₂ to biopterin, to eNOS, and, after further oxidation, to pterin or dihydroxanthopterin. Taken together, our findings suggest that BH₄ is a regulator of vascular redox state and, by decreasing superoxide formation, is further protected from oxidative degradation.

In contrast to the positive associations between vascular biopterin levels, reduced vascular superoxide, and improved endothelial function, we found no similar associations with plasma BH₄. Rather, plasma biopterin levels were associated with CRP, a marker of systemic inflammation. This observation is in agreement with the fact that plasma neopterin, a by-product of BH₄ biosynthesis, is well established as a marker of inflammation.²² Indeed, neopterin was raised in parallel with BH₄ after cytokine stimulation of endothelial cells.²³ Of particular importance for our findings, several previous studies have shown that patients with CAD have elevated plasma neopterin levels.²⁴ Elevated plasma neopterin is associated with accelerated CAD progression¹¹ and increased clinical events, which are also characterized by elevated CRP.¹² Our study now clarifies the implication of these observations because we show that increased plasma biopterin levels are associated with plasma CRP in a manner similar to plasma neopterin but are also associated with
reduced vascular biopterin levels and reduced NO-mediated endothelial function, which would be expected to increase CAD progression and risk.

Despite the fact that endothelial BH₄ is an important determinant of eNOS activity, little is known about the pathophysiological control of endothelial BH₄. In mammalian cells, the biosynthesis of BH₄ begins with GTPCH, which catalyzes the rearrangement of GTP to dihydoropterin triphosphate, which is subsequently converted to BH₄ by the sequential action of 6-pyruvotetrahydrobiopterin synthase and sepiapterin reductase. In contrast to the latter 2 enzymes, GTPCH activity is limiting in most tissues, and it is the major regulator of BH₄ synthesis. The GCH1 gene, encoding GTPCH, is expressed in several cell types such as macrophages, hepatocytes, and endothelial cells. Several in vitro studies suggested that GCH1 expression in endothelial cells or macrophages is induced by cytokines. However, GCH1 upregulation in human endothelial cells requires simultaneous exposure to high concentrations of multiple cytokines (such as interleukin-1β, interleukin-6, tumor necrosis factor-α, and interferon-γ) and lipopolysaccharide, which may not be clinically relevant. Indeed, our data suggest that in patients with CAD, systemic inflammatory stimuli that are sufficient to increase plasma biopterins (in...
A limitation of the present study is the absence of any information about biopterin levels in vessels from healthy individuals. By definition, these measurements require access to surgical material obtained during CABG, and no measurement in healthy human vessels is possible. In addition, this study presents an association between BH₄ and vascular superoxide production/endothelium-dependent vasodilation, which does not prove a cause-and-effect relationship. Finally, it would be interesting to determine whether vascular BH₄ is correlated with the eNOS protein levels in human vessels. However, the high variability and technical limitations of Western blotting make quantitative comparisons in these small vessels difficult.

In conclusion, we demonstrate that vascular BH₄ levels are a key determinant of eNOS coupling, vascular superoxide production, and endothelium-dependent vasodilation in vessels from patients with atherosclerosis. In contrast, plasma biopterin levels are associated with plasma CRP levels and inversely correlated with vascular biopterins and with vascular endothelial function. Discordant regulation of vascular and plasma biopterins in human atherosclerosis provides important mechanistic insights into the relationships between inflammation, endothelial dysfunction, and vascular oxidative stress, with direct implications for therapeutic strategies aimed at improving endothelial function.

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Disclosures
None.

References
Endothelial nitric oxide synthase, which is the main source of nitric oxide in vascular endothelium, may be uncoupled in the absence of its cofactor tetrahydrobiopterin (BH4). However, the extent to which vascular and/or systemic BH4 levels are altered in human atherosclerosis and the importance of BH4 bioavailability in determining endothelial function and oxidative stress remain unclear. In the present study we define the relationships between plasma and vascular biopterin levels in patients with coronary artery disease, and we determine how BH4 levels affect endothelial function, endothelial nitric oxide synthase coupling, and vascular superoxide production in saphenous veins and internal mammary arteries obtained during coronary artery bypass grafting. We demonstrate an inverse association between plasma and vascular biopertin levels in patients with coronary artery disease. We also support that vascular but not plasma BH4 is an important determinant of endothelial nitric oxide synthase coupling, endothelium-dependent vasodilation, and superoxide production in human vessels. On the other hand, plasma biopertin are a marker of systemic inflammation, being positively correlated with C-reactive protein levels in human circulation. These findings suggest a distinct and contrasting biological importance for systemic versus plasma biopertin in patients with coronary artery disease. Vascular biopertin are associated with maintained endothelial nitric oxide synthase coupling and endothelial function, whereas plasma biopertin are associated with inflammation and impaired endothelial function.

**CLINICAL PERSPECTIVE**


Altered Plasma Versus Vascular Biopterins in Human Atherosclerosis Reveal Relationships Between Endothelial Nitric Oxide Synthase Coupling, Endothelial Function, and Inflammation
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