Tetrahydrobiopterin Restores Endothelial Function in Long-Term Smokers
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OBJECTIVES
We sought to test whether tetrahydrobiopterin (BH4) supplementation improves nitric oxide (NO) bioactivity in smokers.

BACKGROUND
In smokers, endothelium-derived NO bioactivity is impaired. BH4 is an essential cofactor of NO synthase, and its deficiency decreases NO bioactivity.

METHODS
Sapropterin hydrochloride, an active analogue of BH4 (2 mg/kg body weight), was administered orally to healthy male smokers and age-matched nonsmokers. Before and 3 and 24 h after sapropterin, we measured plasma levels of BH4 and examined flow-mediated vasodilation (FMV) of the brachial artery by high resolution ultrasonography, a noninvasive test of endothelial function.

RESULTS
Basal plasma levels of BH4 were not different between smokers and nonsmokers. Sapropterin administration increased plasma levels of BH4 by threefold at 3 h, which returned to the baseline at 24 h. Before sapropterin, FMV was significantly smaller in smokers (p = 0.0002). Sapropterin significantly augmented endothelium-dependent vasodilation in smokers, but did not affect it in nonsmokers (p = 0.001 by analysis of variance [ANOVA]). Coadministration of N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor (20 \textmu mol), into the brachial artery completely abolished the vasodilatory effects of sapropterin (p = 0.002 by ANOVA). Endothelium-independent vasodilation by glyceryl trinitrate was not different between smokers and nonsmokers and was not altered by BH4.

CONCLUSIONS
We demonstrated that BH4 supplementation improved bioactivity of endothelium-derived NO in smokers. These observations strongly suggest that decreased NO-dependent vasodilation in smokers could be related to reduced bioactivity of BH4. (J Am Coll Cardiol 2000; 35:71–5) © 1999 by the American College of Cardiology

During the formation of nitric oxide (NO) and L-citrulline, nitric oxide synthase (NOS) requires several cofactors, including NADPH, calcium-calmodulin, flavin nucleotides and tetrahydrobiopterin (BH4) (1,2). Among these cofactors, BH4 plays a crucial role in the regulation of NOS activity. Depletion of BH4 causes uncoupling of the L-arginine–NO pathway, which results in decreased formation of NO and increased formation of oxygen radicals by NOS in vitro (3,4). Recent reports have demonstrated that exogenous administration of BH4 restores impaired NO bioactivity in hypercholesterolemic humans (5) and hypertensive rats (6), suggesting the relative depletion of BH4 in the presence of coronary risk factors.

It has been suggested that decreased bioactivity of endothelium-derived NO through oxidative stress is responsible for endothelial dysfunction in smokers (2,7,8). Although the precise mechanisms are unknown, dysregulation of BH4 may be involved. Higman et al. (9) demonstrated that exogenous BH4 improved endothelium-dependent vasodilation of saphenous vein strips from smokers. However, because this experiment was conducted ex vivo using vein strips, the in vivo effects of BH4 on arterial endothelial dysfunction in smokers need to be elucidated.

In this study, we examined the effects of exogenous BH4 on endothelium-dependent vasodilation in the brachial artery in long-term smokers in the presence or absence of an NOS inhibitor.

METHODS
Subjects. Studies were performed in 17 healthy male volunteers (10 smokers and 7 nonsmokers). None had clinical signs of atherosclerosis on physical examination. Smokers were defined as current smokers consuming at least 20 cigarettes per day (28.6 ± 2.0 cigarettes per day, range 20 to
40) for one year or more (11.6 ± 1.7 years, range 5 to 22), and their smoking status was confirmed by measurement of plasma cotinine (0.10 ± 0.03 µg/ml vs. undetected). Age-matched control subjects were life-long nonsmokers (30 ± 2 vs. 29 ± 1 years old, p = NS). All subjects were not obese (body mass index: 22 ± 1 vs. 23 ± 1 kg/m², p = NS), normotensive (mean arterial pressure: 84 ± 2 vs. 84 ± 2 mm Hg, p = NS), nondiabetic (fasting plasma glucose: 94 ± 5 vs. 98 ± 2 mg/dl, p = NS; glycosylated hemoglobin [HbA1c]: 4.7 ± 0.4% vs. 5.0 ± 0.4%, p = NS) and normolipidemic (total cholesterol: 182 ± 9 vs. 178 ± 10 mg/dl, p = NS; low density lipoprotein [LDL] cholesterol: 108 ± 9 vs. 109.8 ± 8 mg/dl, p = NS; high density lipoprotein [HDL] cholesterol: 51 ± 3 vs. 51 ± 3 mg/dl, p = NS; triglycerides: 115 ± 22 vs. 89 ± 23 mg/dl, p = NS), and there were no significant differences between smokers and nonsmokers in other backgrounds. The subjects had not taken any medications before the experiment.

**Study design.** The protocol was explained, and written informed consent was obtained from each subject. The study was approved by the Ethical Committee for Human Investigation at our institution. The study was performed in the morning after the subjects had fasted overnight in a supine position in an air-conditioned room with room temperature ~25°C. Smokers had abstained from smoking for >120 min before the start of the experiments to avoid the short-term effects of smoking. The fasting blood level was drawn for measurement of plasma cotinine and BH4. We measured blood pressure, heart rate, endothelial function and plasma levels of BH4 before and 3 and 24 h after oral administration of sapropterin hydrochloride, an active form of BH4 (2 mg/kg body weight, Biopent, Suntory, Tokyo). To examine the possible relation between NO and the effects of BH4, in separate occasions under pretreatment with N⁶-monomethyl-L-arginine (L-NMMA) (Clinalfa, Switzerland), a NOS inhibitor, we repeated blood sampling and measurement of vascular function before and 3 h after oral administration of sapropterin hydrochloride in smokers. L-NMMA was infused into the brachial artery with a 22-gauge catheter, placed at least 3 cm proximal to the probe of ultrasonography at a dose of 4 µmol/min for 5 min (20 µmol in total), 15 min before measurement of endothelial function. The catheter was pulled out after infusion. Subjects were not allowed to eat until the 3-h experiment was finished, and measurements at 24-h were conducted after overnight fasting to avoid the effects of diet.

**Measurement of endothelial function.** With the use of high resolution ultrasonography (SSA-380A, Toshiba, Tokyo), the brachial artery was imaged longitudinally just above the antecubital fossa, three times at baseline, and then scanned continuously for 15 min after release of 4.5 min of upper arm arterial occlusion with a 12.5-cm-wide blood pressure cuff that was placed 3 to 5 cm proximal to the studied part (10). To assess endothelium-independent vasodilation, sublingual glyceryl trinitrate (GTN)-induced (300 µg; Myocol Spray, Toa Eiyu Co., Tokyo, Japan) vasodilation was examined. Measurements were conducted in a blinded manner and the interobservational and intraobservational variations of the baseline measurements were 2.8% and 1.6%, respectively.

**Chemical analysis.** Serum total cholesterol and triglyceride levels were determined enzymatically with commercial kits (L-type Wako cholesterol, L-type Wako TG-H, respectively; Wako Chemicals Co., Osaka, Japan). High density lipoprotein cholesterol was determined by homogeneous HDL cholesterol assay with commercial kits (Cholesterol HDL; Daiichi Chemicals Co., Tokyo, Japan). Low density lipoprotein cholesterol was calculated by the Friedewald formula. Plasma glucose was measured by the glucose dehydrogenase ultraviolet test (Merck Liquid Glu; Kanto Chemical Co., Tokyo, Japan). Then HbA1c was measured by latex agglutination turbidimetry with a commercial kit (Rapidea Auto HbA1c; Fujirebio Inc., Tokyo, Japan). Tetrahyrobiopterin was measured by high performance liquid chromatography with the electrochemical detection method, as previously described (11). Cotinine was measured by high performance liquid chromatography.

**Statistical analysis.** Statistical comparisons were performed by two-way analysis of variance (ANOVA) for repeated measures, followed by Bonferroni post hoc analysis for changes in endothelial function and chemical variables before and after BH4 loading. All data are expressed as the mean value ± SEM, and p < 0.05 was considered to be statistically significant.

**RESULTS**

**Vascular function at baseline.** For the baseline vessel diameter of the brachial artery there was no significant difference between smokers and nonsmokers (Table 1). Flow-mediated vasodilation (FMV) of the brachial artery at baseline was significantly smaller in smokers (p = 0.0002, Fig. 1, top). Glyceryl trinitrate–induced brachial artery dilation (p = NS, Fig. 1, bottom) and the magnitude of
reactive hyperemia (409 ± 59% vs. 463 ± 49%, p = NS) were not different between smokers and nonsmokers.

**Effects of BH4 on systemic hemodynamic data.** Basal plasma levels of BH4 were not different between smokers and nonsmokers (Table 1). Sapropterin administration increased plasma levels of BH4 by threefold at 3 h, which returned to the baseline at 24 h in smokers and nonsmokers in a similar manner (Table 1). Oral sapropterin administration did not alter blood pressure and heart rate (Table 1).

**Effects of BH4 on vascular function.** The baseline vessel diameter of the brachial artery was not altered by BH4 in smokers and nonsmokers (Table 1). In smokers, sapropterin significantly restored FMV during reactive hyperemia and endothelium-dependent vasodilation to the near-normal level and returned to the baseline at 24 h (p = 0.0001 by post hoc analysis), although FMV in nonsmokers was not altered by BH4 (p = NS; p = 0.001 for the interactions by ANOVA) (Fig. 1, top). The magnitude of reactive hyperemia (smokers: before test 409 ± 59%; at 3 h 518 ± 64%; at 24 h 471 ± 34%, p = NS—nonsmokers: before test 463 ± 49%; at 3 h 429 ± 42%; at 24 h 422 ± 23%, p = NS—smokers vs. nonsmokers, p = NS) or GTN-induced endothelium-independent vasodilation (Fig. 1, bottom) was not different between smokers and nonsmokers and was not altered by sapropterin.

**Effects of NOS inhibition on the effects of BH4 in smokers (Fig. 2).** Before sapropterin, local infusion of L-NMMA into the brachial artery did not affect blood pressure, heart rate, baseline vessel size (Table 2), the magnitude of reactive hyperemia (before test 388 ± 75%; after test 395 ± 25%, p = NS) or GTN-induced vasodilation, but it significantly decreased FMV (p = 0.0008 by post hoc analysis) (Fig. 2, top). Pretreatment with L-NMMA completely abolished the increase in FMV induced by sapropterin (p = 0.002 for the interactions by ANOVA) (Fig. 2, top), but did not affect GTN-induced vasodilation (Fig. 2, bottom) or the magnitude of reactive hyperemia (before test 395 ± 25%; at 3 h 438 ± 74%, p = NS).

![Figure 1. Effects of oral sapropterin hydrochloride, an active analogue of tetrahydrobiopterin, on flow-mediated (top) and GTN-induced (bottom) vasodilation in smokers and nonsmokers.](image-url)
DISCUSSION

The salient findings of this study are 1) impaired endothelium-dependent vasodilation in long-term smokers was improved by supplementation with BH4; and 2) an inhibitor of NOS completely abolished the vasodilatory effects of BH4.

Endothelial dysfunction in smokers. To assess endothelial function, we measured FMV of the brachial artery by high resolution ultrasonography. As shown in Figure 2, FMV was abolished after L-NMMA infusion, supporting the notion that NO is responsible for FMV of the brachial artery (12). In agreement with previous observations (13–15), we found that FMV was impaired in smokers whose smoking status was verified by plasma cotinine. Because we enrolled subjects without other coronary risk factors and age was matched, smoking could be solely responsible for impaired FMV in smokers. In the present study, the increases in blood flow during reactive hyperemia were similar between smokers and nonsmokers, indicating that the amount of shear stress to the brachial artery during reactive hyperemia was comparable. In addition, we found no difference in GTN-induced vasodilation, a measure of endothelium-independent vascular function, between the two groups. Collectively, the impaired FMV in smokers could be attributed to endothelial dysfunction, but not to the difference in the amount of shear stress or in the reactivity of vascular smooth muscle cells. The precise mechanisms of smoking-related endothelial dysfunction are unknown and may be multifactorial; it may be caused by decreased endothelial NO formation (14,15) or by increased inactivation by reactive oxygen species such as superoxide anion (14,16).

Effects of BH4 on endothelial function. Tetrahydrobiopterin augments NO formation and inhibits superoxide production from NOS in vitro (3,4). It has been suggested that oxidative stress oxidizes intracellular BH4 to dihydrobiopterin, an inactive form of biopterin (1,3). Accordingly, we hypothesized that the decreased bioactivity of NO in smokers may be attributed to the relative deficiency of BH4. To test this hypotheses, we administered BH4 into smokers and examined endothelial function. Administration of BH4 restored FMV only in smokers. Furthermore, pretreatment with an NOS inhibitor completely abolished the vasodilatory effects of BH4, suggesting that BH4 may have augmented endothelial NO production during shear stimuli in smokers. Although the basal and postload plasma levels of BH4 were comparable between smokers and nonsmokers, it is possible that BH4 within the endothelium may be deficient, especially during shear stimulation in smokers.

Study limitations. In this study, we examined endothelial function in the brachial artery but not in the coronary artery, so the clinical significance of the study remains to be

Table 2. Baseline Vessel Diameters and Systemic Hemodynamic Data Before and After L-NMMA Infusion in the Presence or Absence of Tetrahydrobiopterin

<table>
<thead>
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<th>BH4 (-)</th>
<th></th>
<th>p Value</th>
<th></th>
<th>BH4 (+)</th>
<th></th>
<th>p Value</th>
<th>Effects of BH4</th>
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</thead>
<tbody>
<tr>
<td>Vessel diameter (mm)</td>
<td>Before L-NMMA</td>
<td>4.6 ± 0.1</td>
<td>4.7 ± 0.2</td>
<td>NS</td>
<td>After L-NMMA</td>
<td>4.9 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>NS</td>
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<td>Blood pressure (mm Hg)</td>
<td>115 ± 3/69 ± 2</td>
<td>112 ± 3/71 ± 3</td>
<td>NS</td>
<td>111 ± 7/72 ± 4</td>
<td>109 ± 4/71 ± 5</td>
<td>NS</td>
<td>68 ± 5</td>
<td>70 ± 5</td>
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<td>Heart rate (beats/min)</td>
<td>75 ± 3</td>
<td>72 ± 5</td>
<td>NS</td>
<td>115 ± 3/69 ± 2</td>
<td>112 ± 3/71 ± 3</td>
<td>NS</td>
<td>68 ± 5</td>
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Data are expressed as the mean value ± SEM.

BH4 = tetrahydrobiopterin; L-NMMA = N⁵-monomethyl-L-arginine.

Figure 2. Effects of pretreatment with L-NMMA, a NOS inhibitor, on BH4-induced vasodilation in smokers. Infusion of L-NMMA into the brachial artery significantly decreased FMV in smokers (top) (p = 0.0008 by post hoc analysis), but did not affect GTN-induced vasodilation (bottom). The increase (top) (p = 0.0007 by post hoc analysis) in FMV induced by sapropterin 3 h after administration was abolished by pretreatment with L-NMMA (before vs. after sapropterin, p = 0.002 for the interactions by ANOVA).
determined. The relevance of endothelial function in the brachial artery to coronary vascular reactivity in humans exists; it has been shown that endothelial function is impaired not only in the coronary artery but also in the brachial artery in patients with coronary artery disease (17,18). The number of subjects enrolled in this study was relatively small.

Previous studies demonstrated that exogenous administration of BH4 restores impaired NO bioactivity in hypercholesterolemic humans (5) and hypertensive rats (6), in which overproduction of oxygen-derived free radicals as well as impaired NO bioactivity has been reported (2). Thus, biologic depletion of BH4 in the endothelium may be present not only in smokers but also in patients with other coronary risk factors. Orally active sources of BH4 would be a novel therapeutic tool for endothelial dysfunction in humans.

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