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Research Article

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Tetrahydrobiopterin Restores Endothelial Function in Hypercholesterolemia

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Abstract

In hypercholesterolemia, impaired nitric oxide activity has been associated with increased nitric oxide degradation by oxygen radicals. Deficiency of tetrahydrobiopterin, an essential cofactor of nitric oxide synthase, causes both impaired nitric oxide activity and increased oxygen radical formation. In this study we tested whether tetrahydrobiopterin deficiency contributes to the decreased nitric oxide activity observed in hypercholesterolemic patients. Therefore, L-mono-methyl-arginine to inhibit basal nitric oxide activity, serotonin to stimulate nitric oxide activity, and nitroprusside as endothelium-independent vasodilator were infused in the brachial artery of 13 patients with familial hypercholesterolemia and 13 matched controls. The infusions were repeated during coinfusion of L-arginine (200 μg/kg/min), tetrahydrobiopterin (500 μg/min), or the combination of both compounds. Forearm vasomotion was assessed using forearm venous occlusion plethysmography and expressed as ratio of blood flow between measurement and control arm (M/C ratio). Tetrahydrobiopterin infusion alone did not alter M/C ratio. Both the attenuated L-mono-methyl-arginine–induced vasoconstriction as well as the impaired serotonin-induced vasodilation were restored in patients during tetrahydrobiopterin infusion. Tetrahydrobiopterin had no effect in controls. In conclusion, this study demonstrates restoration of endothelial dysfunction by tetrahydrobiopterin suppletion in hypercholesterolemic patients. (*J. Clin. Invest.* 1997. 99:41–46.) Key words: atherosclerosis • nitric oxide • tetrahydrobiopterin • hypercholesterolemia • L-arginine

Introduction

Evidence is accumulating that nitric oxide (NO)[1] is a major determinant of the antiatherosclerotic properties of the endothelium (1, 2). All major risk factors for atherosclerotic vascular disease, including hypercholesterolemia, have been associated with impaired NO activity (3–8). The underlying defect in impaired NO activity may involve both decreased formation (9) as well as increased degradation of NO, by reaction with superoxide (10). Indeed, in several in vitro models increased oxygen radical production has been demonstrated during hypercholesterolemia (11–13). It was proposed recently that enhanced oxygen radical production may be caused by nitric oxide synthase (NOS) itself. This is supported by observations showing that both deendothelialization (12) and infusion of the selective NOS antagonist, nitro-L-arginine (13), could not only prevent NO formation, but could also inhibit increased formation of oxygen radicals. In the presence of a deficiency of tetrahydrobiopterin (BH4), an essential cofactor of NOS (14, 15), uncoupling of the L-arginine–NO pathway is observed, which results in increased formation of oxygen radicals by NOS in vitro (15, 16). In addition, BH4 depletion has been shown to decrease NO production (9). Therefore, we hypothesized that decreased NO-dependent vasodilation in hypercholesterolemia could be related to a relative deficiency of BH4, resulting in the aforementioned functional disturbance of NOS. If so, this hypothesis may provide the basis for a novel therapeutic approach to early atherosclerosis.

In this study the first objective was to observe whether BH4 could improve basal and/or receptor-mediated NO activity in hypercholesterolemic patients. Therefore, we investigated the L-mono-methyl-arginine (L-NMMA)-induced vasoconstrictor response and the serotonin (5-HT)-induced vasodilator response both before and after BH4 infusion. The second objective was to observe whether BH4 infusion could improve the altered enzyme kinetics of NOS. In hypercholesterolemia, L-arginine has been shown to be rate limiting in the L-arginine–NO pathway (3, 17). Presently, we investigated whether infusion of L-arginine still exerted an ameliorative effect on NO activity after suppletion of BH4.

Methods

Patients. Studies were performed in 13 patients (9 males), mean age 32 (4) yr, with definite familial hypercholesterolemia, characterized by LDL-cholesterol of 4.5 mmol/liter or more, VLDL-cholesterol of 2.0 mmol or less, and a family history of premature atherosclerosis and/or tendon xanthomata (18). In the majority of these familial hypercholesterolemia patients, moreover, a molecular diagnosis was established. None had clinical signs of atherosclerosis on physical examination and ECG. Patients did not smoke. Mean plasma cholesterol averaged 8.7 (1.2) mmol/liter. Mean arterial pressure was 85 (2) mmHg, body mass index was 25.0 (0.9) kg/m², and forearm volume was 1,201 (66) ml. Control studies were performed in 13 sex- (9 males) and age-matched [mean age 28 (2) yr] control subjects. Control subjects did not smoke. Mean plasma cholesterol was 4.0 (0.5) mmol/liter. Mean arterial pressure averaged 82 (2) mmHg, body mass index was 23.4 (0.6) kg/m², and mean forearm value was 1,181 (58) ml.

1. *Abbreviations used in this paper:* BH4, tetrahydrobiopterin; FBF, forearm blood flow; 5-HT, serotonin; L-NMMA, L-mono-methyl-arginine; M/C ratio, ratio of forearm blood flow between measurement and control arm; NO, nitric oxide; NOS, nitric oxide synthase; SNP, sodium nitroprusside.

Protocol. Vascular function in the forearm was studied 2 wk after cessation of maintenance lipid-lowering medication in patients. 12 h before the forearm studies, all subjects refrained from drinking alcohol or caffeine-containing beverages. The study protocol was approved by Utrecht University Hospital Ethics Committee for study in human beings. Patients and subjects all gave written informed consent after explanation of the protocol.

The experiments were performed in a temperature controlled room (22–24°C). Subjects were in supine position with both forearms resting slightly above the level of the heart. A 22-gauge needle was inserted into the brachial artery after local anesthesia. Forearm blood flow (FBF) was measured in both arms at 15-s intervals using strain–gauge, venous occlusion plethysmography with a microcomputer-based R-wave triggered system for on-line, semicontinuous monitoring (19). Upperarm cuffs were inflated to 45 mmHg. During FBF measurement the hands were excluded from the circulation by wrist cuffs, inflated 40 mmHg above systolic blood pressure. Baseline measurements were performed at least 45 min after cannulation of the brachial artery. Between infusions, a break of at least 20 min was applied to allow FBF to recover before each subsequent infusion. The following substances were infused: 5-HT (Sigma Chemical Co., St. Louis, MO), sodium nitroprusside (SNP; Merck, Darmstadt, Germany), L-arginine hydrochloride (Bufa, Uitgeest, The Netherlands), L-NMMA (Sigma Chemical Co.), and (6R)-5,6,7,8-tetrahydro-L-biopterindihydrochloride (BH4; Alexis Corp., Laufelfingen, Switzerland). All solutions were prepared aseptically from sterile stock solutions or ampoules on the day of the study and stored at 4°C until use. BH4 was prepared freshly just before administration using oxygen-free saline.

The infusion schemes are depicted in Fig. 1. In a subgroup of six patients (four males) and six age- and sex-matched controls, 5-HT was administered in a cumulative dose infusion of 0.1 (dose I), 0.3 (dose II), and 1.0 (dose III) ng/kg/min (scheme 1, block A) (3). Steady state vasorelaxation, due to NO release, was reached after $\sim$ 3.5 min; each dose–infusion lasted for 5 min. Endothelium-independent vasodilation was assessed by infusion of SNP in a cumulative dose of 1 (dose I), 10 (dose II), 30 (dose III), and 100 (dose IV) ng/kg/min (scheme 1, block B) (3). Steady state vasorelaxation was reached after 1.5 min; each dose–infusion lasted for 3 min. Basal NO activity was estimated by assessment of vasoconstrictor response to cumulative dose infusion of L-NMMA at a dose of 7.5 (dose I), 15 (dose II), and 30 (dose III) µg/kg/min for 5 min per dose (scheme 1, block C), where the latter has been shown to cause near maximal arterial contraction (20, 21). In the first infusion scheme, infusion blocks A and B were infused in randomized order, followed by block C. Next, BH4 (scheme 1, block D) was infused at a rate of 10 (dose I), 100 (dose II), 500 (dose III), and 1,000 (dose IV) µg/min for 5 min per dose. These dosages were chosen to achieve calculated plasma concentrations in the forearm (3, 22, 23) of, respectively, 1, 10, 50, and 100 µM, since in in vitro research maximal NO production by endothelial NOS (NOS-III) was reached at BH4 concentrations ranging from 10 to 100 µM (24). Finally, the first three infusion blocks were repeated during coinfusion of BH4 (dose III = 500 µg/min) (scheme 1, blocks E–G).

In a subgroup of seven patients (five males) and seven age- and sex-matched controls a slightly modified infusion scheme was used to assess whether L-arginine during BH4 infusion had an additional effect on endothelium-dependent vasomotion and to find out whether an effect of BH4 remained or disappeared after cessation of BH4 infusion. L-arginine was infused at a dose of 200 µg/kg/min, which has been reported to enhance metacholine-stimulated endothelial vasodilation in hypercholesterolemia (25). Thus, after infusion of SNP with saline (Fig. 1, scheme 2, block A), 5-HT was infused during coinfusion of, respectively, saline (block B), L-arginine (block C), BH4 (block D), and BH4 in combination with L-arginine (block E). Next, SNP infusion was repeated during coinfusion of BH4 in combination with L-arginine (block F). Finally, after cessation of both BH4 and L-arginine infusion for 20 min, 5-HT infusion was repeated during coinfusion of saline (block G).
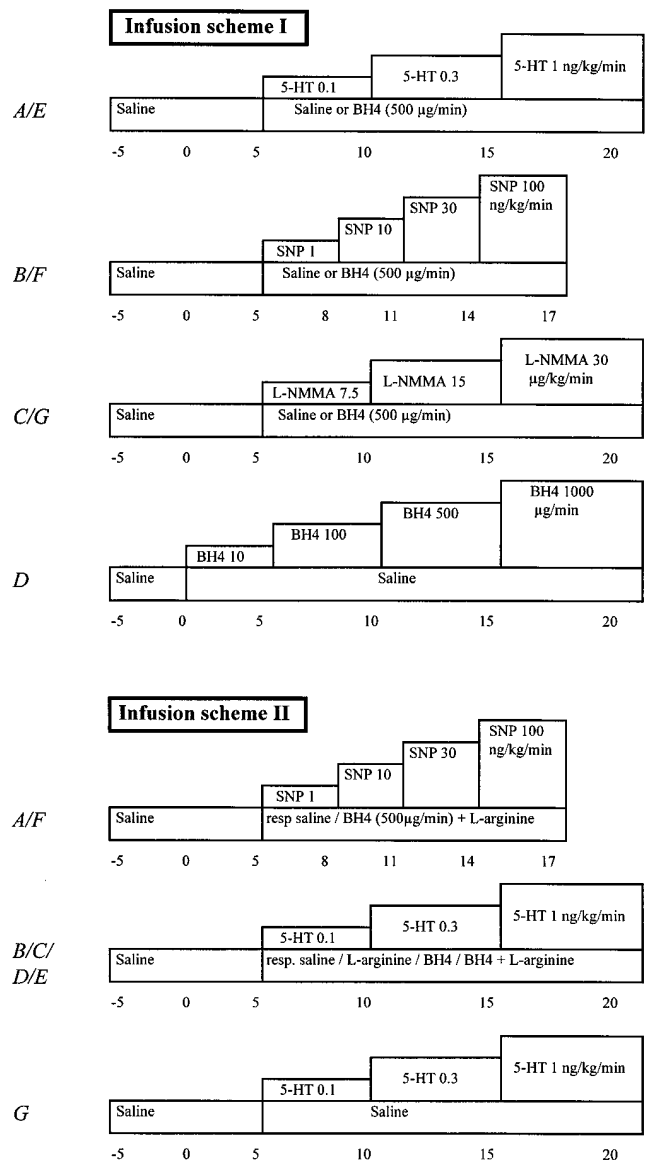
Figure 1. Infusion schemes. (*Infusion scheme 1*) Cumulative dose infusions of 5-HT (*A/E*), SNP (*B/F*), and the NOS inhibitor, L-NMMA (*C/G*), were performed during, respectively, saline (*A/B/C*) and BH4 (*E/F/G*) coinfusion. (*Infusion scheme 2*) Cumulative dose–infusion of SNP (*A*) was followed by cumulative dose–infusion of 5-HT during coinfusion of, respectively, saline (*B*), L-arginine (*C*), BH4 (*D*) and BH4 with L-arginine (*E*). Then, cumulative dose–infusion of SNP (*F*) was repeated during BH4 infusion with L-arginine. Finally, $\sim$ 20 min after cessation of L-arginine and BH4 infusion, cumulative dose–infusion of 5-HT was repeated (*G*).

Analysis. FBF was expressed as ml/100 ml forearm tissue/min. During each infusion step the final six values of FBF from both measurement and control arm were used to calculate the mean FBF and the ratio of FBF between measurement and control arm (M/C ratio [26]). From the subsequent M/C ratio, curves were constructed (Figs. 2–4). Results are expressed as means (SE).

Differences between M/C ratio curves during coinfusion of saline versus, respectively, BH4, L-arginine, or the combination of latter compounds, were tested using a repeated measures two-way ANOVA. Differences in M/C ratio curves between patients and controls were evaluated using two-way ANOVA where the interaction ratio indicated differences between curves. Alterations in absolute FBF upon
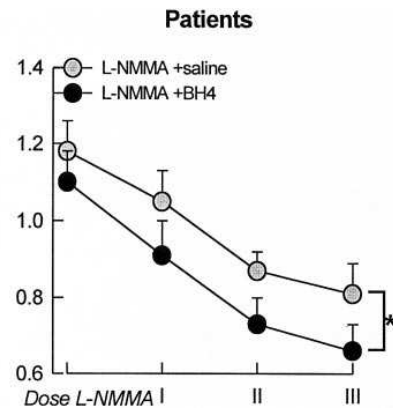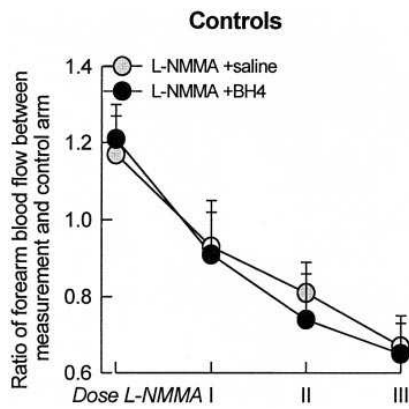
Figure 2. Effect of BH4 on basal NO activity. The decrease in M/C ratio upon L-NMMA infusion was unaltered by BH4 in controls ($n = 13$). In patients ($n = 13$), L-NMMA during BH4 induced a larger reduction in M/C ratio compared with L-NMMA during saline. *$P < 0.05$ L-NMMA response curve during saline vs. during BH4.

infusion of the various agonists compared with baseline FBF were tested using one-way repeated measures ANOVA. If variance ratios reached statistical significance, differences between the means were analyzed with the Student-Newman-Keul's test for $P < 0.05$.

Results

Influence of BH4 on baseline vasomotion. Upon cumulative dose–infusion of BH4, basal FBF was not significantly altered in either patients ($n = 6$) or control subjects ($n = 6$) [flow patients from 2.6 (0.4) to 2.8 (0.4) (NS); in controls from 2.5 (0.3) to 2.7 (0.4) (NS) ml/100 ml forearm tissue/min; both not significantly different from baseline]. Accordingly, the M/C ratios were unaltered by BH4 coinfusion.

Influence of BH4 on endothelium-dependent, basal NO activity. In control subjects ($n = 6$) the decrease in FBF upon

L-NMMA infusion (30 µg/kg/min) was 38 (3)% during saline [FBF from 3.0 (0.4) to 1.9 (0.3) ($P < 0.05$) ml/100 ml forearm tissue/min] compared with 40 (3)% during BH4 [FBF from 3.2 (0.4) to 1.9 (0.2) ($P < 0.05$)] (M/C ratio curves saline vs. BH4 NS; Fig. 2). In patients ($n = 6$) the decrease in FBF upon L-NMMA was 32 (3)% during saline [FBF from 2.9 (0.4) to 2.0 (0.3) ($P < 0.05$)] compared with 41 (2)% during BH4 [FBF from 3.1 (0.4) to 1.8 (0.3) ($P < 0.05$)]. As reflected in the M/C ratio curve (Fig. 2), in patients the vasoconstrictor response to L-NMMA was significantly increased during BH4 compared with saline infusion ($P < 0.05$).

Influence of BH4 on endothelium-dependent stimulated NO activity. In control subjects ($n = 13$) coinfusion of BH4 did not significantly alter 5-HT–induced, NO-mediated vasodilation [saline: FBF 2.5 (0.2) to 6.0 (0.6) ($P < 0.05$); BH4: FBF 2.5 (0.2) to 6.1 (0.7) ($P < 0.05$) ml/100 ml forearm tissue/min]) (M/C
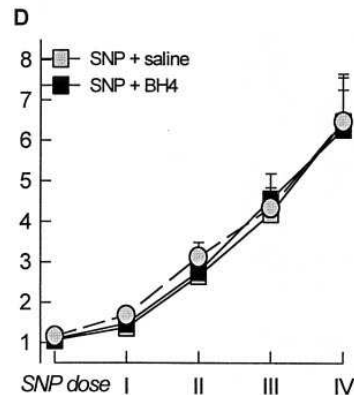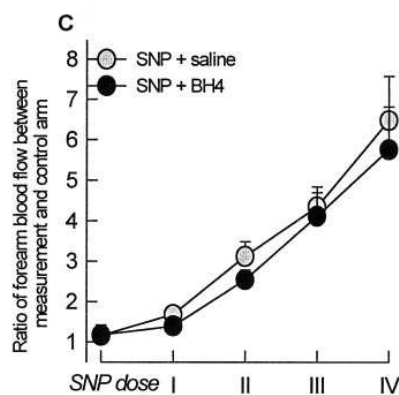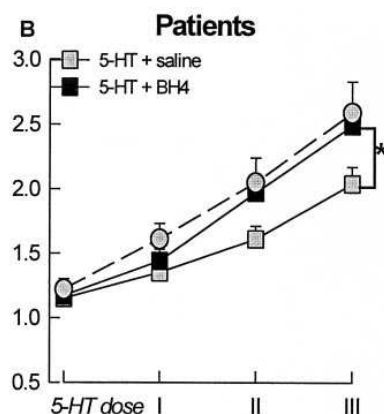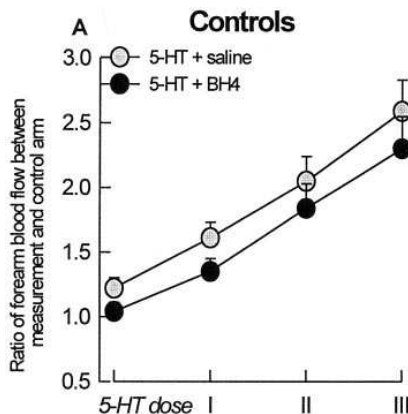

Figure 3. Effect of BH4 on vasodilator responses. (*A* and *B*) Whereas BH4 did not significantly alter 5-HT–induced increase in the M/C ratio in controls (*A*), BH4 significantly enhanced the 5-HT response in patients (*B*). The dotted line in *B* is added as reference value of 5-HT–induced vasodilation in the control group (*A*). (*C* and *D*) BH4 did not alter endothelium-independent responses in either controls (*C*) or patients (*D*). The dotted line in *D* is added as reference value of nitroprusside-induced vasodilation in the control group (*C*). *$P < 0.05$ 5-HT response curve during saline vs. BH4.

ratio curve saline vs. BH4 NS; Fig. 3). In contrast, in patients (*n* = 13) 5-HT–induced vasodilation was significantly enhanced by BH4 coinfusion [saline: FBF 2.9 (0.2) to 5.5 (0.5) (*P* < 0.05); BH4 2.9 (0.3) to 6.7 (0.9) (*P* < 0.05) ml/100 ml forearm tissue/min] (M/C ratio curve saline vs. BH4; *P* < 0.05). Accordingly, whereas the M/C ratio curve for 5-HT in patients was significantly impaired compared with controls (*P* < 0.05), this difference between patients and controls was abolished upon BH4 coinfusion in patients (NS) (Fig. 3).

In the subgroup of seven controls, 5-HT response [from 2.4 (0.3) to 6.3 (1.0) (*P* < 0.05)] was not significantly altered by coinfusion of either L-arginine [from 2.5 (0.3) to 6.2 (1.0) (*P* < 0.05)] or L-arginine in combination with BH4 infusion [from 2.3 (0.3) to 6.3 (1.1) (*P* < 0.05)] (M/C ratio curves saline vs. L-arginine with/without BH4 NS). In the subgroup of seven patients, L-arginine enhanced 5-HT response [saline: from 2.9 (0.3) to 5.8 (0.6) (*P* < 0.05), L-arginine: from 3.0 (0.3) to 6.4 (0.7) (*P* < 0.05) ml/100 ml forearm tissue/min] (M/C ratio curve saline vs. L-arginine, *P* < 0.05; Fig. 4). Coinfusion of L-arginine during BH4 infusion did not have an additional effect on 5-HT–induced vasodilation [from 3.3 (0.4) to 7.2 (1.0) (*P* < 0.05) ml/100 ml forearm tissue/min] compared with BH4 infusion alone [FBF from 2.9 (0.3) to 6.7 (0.9)] (M/C ratio curve BH4 versus BH4 with L-arginine, NS). The beneficial effects of BH4 were transient, since 20 min after cessation of BH4/L-arginine infusion the beneficial effect could no longer be demonstrated [baseline FBF: from 2.9 (0.3) to 5.8 (0.6), cessation of BH4/L-arginine: from 3.1 (0.3) to 6.1 (0.6) ml/100 ml forearm tissue/min] (M/C ratio curve baseline vs. after cessation of BH4/L-arginine NS; Fig. 4).

Influence of BH4 on endothelium-independent vasomotion. SNP response was not significantly different between control subjects and patients (see M/C ratio curves, Fig. 3).

BH4 coinfusion had no effect upon SNP-induced vasodilation in either controls [FBF with saline: 2.7 (0.4) to 15.5 (1.9) (*P* < 0.05), with BH4 3.1 (0.4) to 16.2 (2.2) (*P* < 0.05) ml/100 ml forearm tissue/min] (M/C ratio curves saline vs. BH4 NS) or patients [FBF with saline: 2.4 (0.4) to 14.7 (1.6) (*P* < 0.05), with BH4 3.1 (0.5) to 15.3 (1.7) (*P* < 0.05)] (M/C ratio curve saline vs. BH4 NS; Fig. 3). Additionally, L-arginine infusion during BH4 did not alter SNP response in either controls [FBF with saline 2.3 (0.3) to 14.6 (1.5) (*P* < 0.05), with BH4 + L-arginine 3.2 (0.3) to 15.0 (1.4) (*P* < 0.05)] (M/C ratio curves saline vs. BH4 + L-arginine NS) or patients [FBF with saline: 2.8 (0.3) to 14.2 (1.8) (*P* < 0.05), with BH4 + L-arginine 3.2 (0.3) to 14.2 (1.1) (*P* < 0.05) ml/100 ml forearm tissue/min] (M/C ratio curves saline vs. BH4 + L-arginine NS).

Discussion

In this study it is demonstrated that in hypercholesterolemic patients BH4 restores the disturbed NO-dependent vasodilation. L-arginine improved NO-dependent vasodilation in these patients, whereas L-arginine caused no further improvement in the presence of BH4 suppletion. BH4 and/or L-arginine coinfusion had no effect on endothelium-independent vasodilation in patients, nor on endothelium-dependent and -independent vasodilation in healthy controls.

Effect of BH4 on basal FBF. BH4 infusion by itself did not significantly alter basal FBF in either patients or controls. In in vitro experiments BH4 has been shown to exert both vasorelaxing (27) as well as vasoconstrictor (28) effects. In these experiments possible confounding factors include the induction of inducible NOS, i.e., NOS-II, during preparation and autooxidation of BH4 (29). Notably, NOS-II has been demonstrated
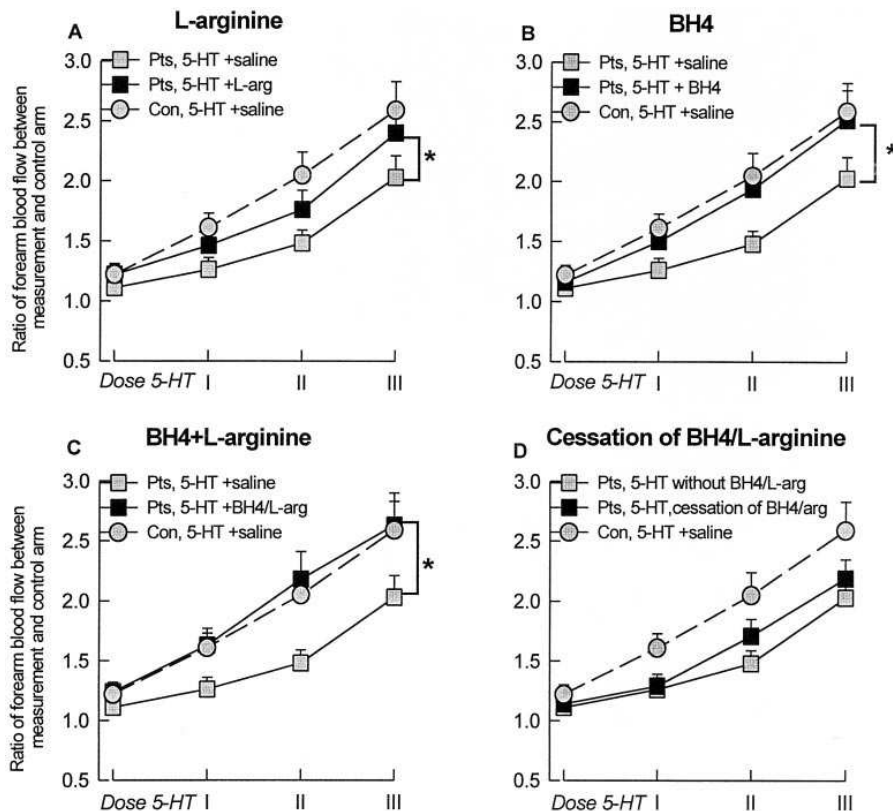
Figure 4. Interaction of BH4 and L-arginine on NO activity. 5-HT–induced increase in the M/C ratio in hypercholesterolemic patients was significantly enhanced by coinfusion of either L-arginine (*A*), BH4 (*B*), and BH4 + L-arginine (*n* = 7) (*C*). 20 min after cessation of BH4/L-arginine infusion, the improvement in endothelial response had almost completely disappeared (*D*). The dotted line is added as reference value of 5-HT–induced vasodilation in the control group. **P* < 0.05; 5-HT response curve during saline vs. during BH4 and/or L-arginine. MC ratio curve BH4 (*B*) versus BH4/L-arg (*C*) not significantly different.

to be critically dependent on BH4 availability (30, 31). One uncontrolled study reported also vasorelaxing effects of BH4 in vivo in two healthy subjects (32). However, in that study up to 50-fold higher dosages were infused.

Effect of BH4 on NO activity. To assess basal NO activity in patients and controls, the vasoconstrictor response to incremental dosages of the specific NOS inhibitor, L-NMMA, was measured. There was a tendency towards a decreased constrictor response to L-NMMA in patients, which did not reach statistical significance compared with healthy controls. However, whereas BH4 coinfusion did not alter the response in healthy volunteers, the vasoconstrictor response to L-NMMA was significantly enhanced in patients by BH4. This is compatible with an, albeit mild, degree of impairment in basal NO activity, which can be improved by BH4 suppletion in hypercholesterolemic patients. We also studied vasodilation during stimulation of NO by 5-HT, which in the dosages used elicits selective NO-mediated vasodilation in both hypercholesterolemic patients (3) as well as in controls (3, 22, 23). Stimulated NO activity was significantly impaired in patients compared with controls, which is in accordance with earlier studies demonstrating impaired endothelial function in patients with hypercholesterolemia (3, 4). Coinfusion of BH4 could restore the impaired NO-dependent vasodilation in patients, but had no such effect in controls. Given the improvement of basal NO activity by BH4, one would also have expected an effect of BH4 on basal FBF. However, it should be noticed that the forearm model has a limited sensitivity to detect changes in basal flow (3, 7, 17). Accordingly, only changes in maximal inhibition and receptor stimulation reached significance.

The action of BH4 proved to be transient, since discontinuation of BH4/L-arginine infusion rapidly resulted in reoccurrence of impaired NO activity. One would expect a longer lasting effect of BH4 since binding of BH4 to NOS is tight (16) and NOS has been shown, once bound, to autoregenerate the inactive oxidized form of BH4, quinonoid dihydrobiopterin, back to the active BH4 (33). One possible explanation for the short duration could be that the increased production of reactive oxygen species that has been demonstrated during hypercholesterolemia (11–13) results in enhanced oxidation of BH4. Since only reduced BH4 is active as cofactor for NOS (34), this would limit the duration of action of BH4 suppletion.

Mechanisms of BH4-induced increase in NO activity in hypercholesterolemia. An improvement in NO activity by BH4 suppletion in hypercholesterolemia signifies either increased formation or decreased degradation of NO. Increased formation of NO upon BH4 suppletion could well be related to the essential role of BH4 as cofactor for the NOS enzyme (34). BH4 increases NOS activity by serving as electron donor for the hydroxylation of L-arginine (14, 35), by serving as allosteric factor, which helps to stabilize the active dimeric state of the NOS enzyme (36, 37), or by inhibiting the direct negative feedback inhibition of NO upon NOS (29, 34). If the increased NO activity can be explained by enhanced NO formation, one has to assume that hypercholesterolemia is accompanied by an absolute or relative deficiency of BH4. The background for such a deficiency is not clear. Perhaps, the enhanced oxidative stress in hypercholesterolemia accelerates oxidation of BH4 (29, 38). As mentioned earlier, this option would also explain the transient effect of BH4 suppletion on NOS activity. BH4 deficiency may also be explained by the observation that in human endothelial cells NOS-III becomes critically dependent upon

BH4 availability in the presence of inflammatory cytokines (39). Interestingly, in vivo hypercholesterolemia (40) and atherosclerosis (41) have been shown to be accompanied by inflammatory changes of the endothelium with local release of cytokines. However, so far this inflammatory response has been established for coronary conduit arteries and it remains to be established whether this also holds true for resistance vessels.

It is not very likely that the observed effect of BH4 can be explained by an effect of BH4 on NOS-II, which may be present in vascular smooth muscle cells and macrophages in the vascular wall and which in vitro has been shown to be dependent upon BH4 concentration (30, 31). In this respect, 5-HT induces a receptor-operated calcium-mediated increase in NOS-III activation, whereas it has no effect on the calcium-independent NOS-II.

Apart from increased NO production by BH4, decreased degradation of NO may also explain the observed increase in NO activity. A main pathway of inactivation of NO involves its reaction with superoxide anion (10). Increased breakdown of NO could also be explained from a BH4 deficiency. During BH4 deficiency, NOS-III has been shown to produce, besides NO, a disproportionate amount of oxygen radicals (15, 16). BH4 suppletion may thus restore NO activity by decreasing oxygen radical formation. Restoration of endothelial function by an NOS-unrelated action of BH4 seems less likely. A recent study showed that BH4 by itself, in the absence of NOS, may in fact generate superoxide (28) and thus decrease NO activity.

BH4 and the effect of L-arginine. L-arginine has been shown to ameliorate impaired NO activity in hypercholesterolemia. Interestingly, in vivo L-arginine concentrations appear to be well above the K_m of NOS for L-arginine (42, 43). The observation that L-arginine seems to be rate-limiting under these conditions, despite saturating concentrations of L-arginine, has been referred to as the "L-arginine paradox" (44). In the present study BH4 suppletion abolished the rate limiting role of L-arginine in hypercholesterolemic patients. Interestingly, BH4 has also been demonstrated to increase substrate affinity of NOS for L-arginine (45, 46). It is tempting to speculate that decreased affinity of NOS for L-arginine due to BH4 deficiency contributes to the L-arginine paradox in hypercholesterolemia.

Summary. Endothelial dysfunction is an event which occurs early in the development of cardiovascular disease, even before the onset of macroscopical structural changes (47–49). In the present study it is shown that endothelial dysfunction can be restored in patients with hypercholesterolemia by BH4 suppletion, at a stage where macrovascular disease has not yet occurred. These data not only underscore the relevance of BH4 as crucial cofactor for NO synthesis in vivo in humans, but also may initiate research into new therapeutic approaches to prevent initiation and progression of cardiovascular disease in hypercholesterolemia.

Acknowledgments

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