Tetrahydrobiopterin corrects *Escherichia coli* endotoxin-induced endothelial dysfunction

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Despite the apparent involvement of redox processes during experimental inflammation, leading to inactivation of vasoactive substances (23), it remains unclear whether functional impairment is caused by the decomposition of nitric oxide (NO) by ROS, or whether it could also be caused by diminished availability of tetrahydrobiopterin (BH4) (6). BH4, a cofactor required for activity of all NO synthase (NOS) isoforms (18, 29), has antioxidant capabilities (32). Reduced intracellular BH4 concentration could shift production of protective NO to deleterious radical formation (6). Several biochemical studies have demonstrated that activation of constitutive NOS in the presence of suboptimal levels of BH4 results in uncoupling of oxygen reduction and arginine oxidation, thereby generating ROS (9, 25, 35). The salutary effects of vitamin C in our previous experiments (23, 24) may partly be explained by protection of BH4 against oxidation (12, 14).

In this study, we therefore tested the hypothesis that exogenous BH4 could improve inflammation-induced endothelial dysfunction. Acute inflammation was induced by administration of *Escherichia coli* endotoxin (LPS), which is an established model for a transient systemic inflammatory response in humans (21, 22, 36). The effect of BH4 on endothelium-dependent and -independent vasodilation in the forearm was studied by strain-gauge plethysmography, and systemic cytokine concentrations were monitored. Administration of vitamin C was selected as a positive control. To examine whether vitamin C may also affect cellular BH4 availability, supernatant and intracellular BH4 concentrations were measured in human umbilical vein endothelial cells (HUVEC) incubated with and without LPS.

**METHODS**

The study was approved by the Ethics Committee of the University of Vienna and conforms to the principles outlined in the Declaration of Helsinki, including current revisions, and the “Good Clinical Practice” guidelines.

**Study population.** Signed informed consent was obtained from eight healthy male subjects (26 ± 3 yr old) before enrollment in this randomized, double-blind, placebo-controlled, three-way crossover study. All subjects were given a complete health examination (including physical examination, ECG, and laboratory screening) before the first study day, were nonsmokers, and had no history or signs of arterial hypertension, hypercholesterolemia, or other cardiovascular disease.

ENDOTHELIAL DYSFUNCTION is present during experimental inflammation in venous (4) and arterial vascular beds in humans (22) and may be a link between acute systemic inflammation and the transiently elevated risk of cardiovascular events (26). The mechanisms of endothelial dysfunction are not fully understood. Oxidative stress with increased generation of reactive oxygen species (ROS) has been implicated in animal and in vitro models (2, 20). We recently demonstrated that inflammation-induced endothelial dysfunction can be abolished by high doses of the antioxidant vitamin C (23).

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risk factors. Subjects were drug free, including “over-the-counter” medications, from 3 wk before screening until the study was completed. Studies were conducted in a quiet room with an ambient temperature of 22°C and complete resuscitation facilities after an overnight fast.

**Forearm blood flow measurements.** Forearm blood flow (FBF) was measured in both arms as described previously (13, 22). Strain gauges, placed on the forearms, were connected to plethysmographs (model EC-6, Hokanson, Bellevue, WA) to measure changes in forearm volume in response to inflation of venous congesting cuffs. Drug effects are expressed as the ratio of blood flow in the intervention arm to that in the control arm (3), where predose ratio is defined as 100%. Wrist cuffs were inflated to suprasystolic pressures during each measurement to exclude circulation to the hands. Flow measurements were recorded for 9 s at 30-s intervals during drug infusions, and the early linear rise of the flow curves was used for calculation of FBF.

**Generation of acute systemic inflammation.** E. coli endotoxin (LPS, 20 IU/kg body wt; National Reference Endotoxin, US Pharmacopeia, Rockville, MD) was administered intravenously as a bolus to induce acute inflammation. Injection of LPS into humans has been established as a model for acute systemic inflammation (21, 22, 36) and causes endothelial dysfunction that is most marked 3.5–5 h after bolus administration. The flulike symptoms are mild and transient, and subjects can be discharged after ~8–10 h in good health without sequelae.

**Study design.** LPS was administered on 3 different study days, 1 wk apart. An intra-arterial infusion of BH4, vitamin C or placebo into the brachial artery was started 205 min after LPS administration in a randomized, double-blind, three-way crossover design on the trial days. Vitamin C, which was shown to normalize inflammation-induced endothelial dysfunction (23), was used as positive control.

**Experimental protocol.** A plastic cannula was inserted into a forearm vein of the control arm for monitoring of white blood cell (WBC), BH4, vitamin C, high-sensitivity C-reactive protein (hsCRP), IL-6, IL-1β, interferon-γ, and monocyte chemotactant protein (MCP)-1 in venous blood as well as for LPS administration and into a forearm vein of the intervention arm for quantification of local BH4 and vitamin C concentrations in the venous effluent during intra-arterial infusions. A fine-bore (27-gauge) needle (Sterican, Braun, Melsungen, Germany) was inserted into the brachial artery of the nondominant arm for local drug administration. After a 20-min rest period, baseline FBF was recorded (5 min), and the response to the endothelium-dependent dilator Ach (25, 50, and 100 nmol/min, each for 3 min; Clinalfa, Läufelfingen, Switzerland) was measured. After a 15-min washout period to allow restoration of baseline blood flow, FBF was measured in response to the endothelium-independent dilator glyceryl trinitrate (GTN; 4, 8, and 16 nmol/min, each for 3 min; Gebro Pharma, Fieberbrunn, Austria).

Then LPS was administered intravenously. At 205 min after LPS, subjects received a continuous intra-arterial infusion of BH4 [500 μg/min (34) for 47 min; Clinalfa], vitamin C [24 mg/min (23) for 47 min; Mayerhofer, Linz, Austria], or saline. At 5 min after the start of the continuous infusion, the responses to coadministration of Ach and GTN were reassessed as described above.

**Monitoring.** Blood for analysis of WBC, BH4, vitamin C, and hsCRP was drawn at baseline and 205 and 320 min after LPS administration. Typanic temperature (Thermoscan pro, Braun) was measured at frequent intervals, and ECG, pulse rate, and blood pressure were recorded with a standard device (CMS patient monitor, Hewlett-Packard, Palo Alto, CA).

**Cell culture experiments.** HUVEC were prepared as described elsewhere (17) and cultured in human fibronectin-coated 75-cm² plastic flasks in RPMI 1640 containing 1% t-glutamine, 100 μg/ml penicillin G, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B (Fungizone) and supplemented with 5% fetal calf serum. Experiments were carried out with confluent monolayers of passages 2–4. HUVEC were incubated with LPS from E. coli serotype 055:B5 (Sigma; 1 μg/ml), vitamin C (100 μM), or LPS and vitamin C for 24 h. The supernatant was collected, and HUVEC monolayers were detached with trypsin-EDTA and resuspended in HEPES buffer (pH 7.4). The cells were pelleted, and 100 μl of oxidant solution (0.02 M KI-1, in 0.1 M HCl) were added. After sonication on ice, aliquots for the determination of proteins were taken, and the homogenates were incubated for 1 h under light protection at room temperature. The precipitates were removed by centrifugation, and excess iodine was destroyed by the addition of 10 μl of vitamin C (0.2 M). Samples were stored at −30°C until analysis. Protein concentrations were determined by the Bradford assay (5). Sets of five separate experiments were performed.

**Laboratory tests.** Analyses for WBC were carried out on a model XE 2100 analyzer (Sysmex Deutschland, Hamburg, Germany). Plasma BH4 concentration was assessed by reverse-phase HPLC with application of a method described previously (16). Briefly, an LPS solution was subjected to oxidation with iodine for 1 h. This process was terminated by addition of vitamin C. Subsequently, the plasma was deproteinized by addition of HCl and separated on a 5-μm HPLC column (LiChropher 100 RP-18, Merck, Darmstadt, Germany) with 20 mM phosphate buffer (pH 6.4) containing 5% methanol as mobile phase. Plasma vitamin C concentration was measured with a commercially available HPLC kit (Immundiagnostik, Bensheim, Germany). hsCRP was measured on a nephelometer (model BN II, Dade-Behring, Marburg, Germany). The sensitivity of the assay is 0.017 mg/dl (27, 28). Cytokines were analyzed on a system that allows the measurement of multiple cytokines from a single plasma sample (model 100, Luminex, Austin, TX) with application of the Bioplex protein array system (Bio-Rad Laboratories, Hercules, CA).

**Statistical analysis.** Hemodynamics and laboratory parameters are expressed as absolute values or percent changes from baseline and compared using Wilcoxon’s matched-pairs test or the Kolmogorov-Smirnov two-sample test as appropriate. The effects of Ach and GTN at baseline and after LPS administration were assessed by two-way ANOVA for repeated measurements after logarithmic transformation using the Statistica software package (release 6, StatSoft, Tulsa, OK). For comparison of BH4 concentrations in cell culture experiments, the Kolmogorov-Smirnov two-sample test was used. P < 0.05 was considered significant. Values are means ± SD unless indicated otherwise.

**RESULTS**

All infusions were well tolerated, and no adverse events were reported. After LPS administration, the expected mild and transient flulike symptoms occurred. The LPS-induced increase in WBC after 205 min (P < 0.02) was paralleled by an increase in pulse rate and temperature (P < 0.02; Table 1). Mean arterial blood pressure did not change significantly (Table 1). Hemodynamic parameters returned to baseline 8 h after LPS administration. Resting baseline FBF was comparable between study days and was not affected by LPS. LPS had no effect on BH4 but significantly decreased vitamin C plasma concentration (P < 0.02; Table 2). hsCRP concentration decreased after 205 min and increased 320 min after LPS administration (P < 0.02; Table 2).

**LPS and cytokine responses.** Plasma concentrations of IL-6, IL-1β, IFN-γ, and MCP-1 increased significantly after LPS on the placebo day: from 18 ± 15 to 680 ± 573 pg/ml, from <0.98 to 4 ± 3 pg/ml, from 10 ± 6 to 28 ± 9 pg/ml, and from 21 ± 6 to 543 ± 110 pg/ml, respectively (all P <
vasodilation was slightly less with BH₄ or placebo, but this difference was not statistically significant. LPS reduced maximum ACh-induced vasodilation was reduced by only 4% (P < 0.02; Table 2). Vitamin C had no effect on LPS-induced changes in hemodynamics, temperature, WBC, platelet count, hsCRP, or FBF. In the presence of vitamin C, maximum ACh-induced vasodilation was reduced by only 0 ± 40% after LPS administration compared with baseline (P = NS; Fig. 3). Again, maximum GTN-induced vasodilation was slightly reduced by 8 ± 28% (P = NS; Fig. 3). This small hyporeactivity was not different from that at baseline or during infusion of placebo or BH₄.

**Effects of vitamin C.** Exogenous vitamin C increased systemic vitamin C concentration (P < 0.02; Table 2) and had no effect on systemic BH₄ concentration. However, local BH₄ concentration in the forearm increased by 32 ± 36% after vitamin C administration during LPS-induced inflammation (P < 0.02; Table 2). Vitamin C had no effect on LPS-induced changes in hemodynamics, temperature, WBC, platelet count, hsCRP, or FBF. In the presence of vitamin C, maximum ACh-induced vasodilation was reduced by only 0 ± 40% after LPS administration compared with baseline (P = NS; Fig. 3). Again, maximum GTN-induced vasodilation was slightly reduced by 8 ± 28% (P = NS; Fig. 3). This small hyporeactivity was not different from that at baseline or during infusion of placebo or BH₄.

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**DISCUSSION**

This study demonstrates that ACh-induced vasodilation is attenuated by experimental inflammation in humans and can be restored by exogenous BH₄. Forearm reactivity to GTN was not significantly influenced by LPS or by the drugs under study. This selective impairment of endothelium-dependent vasodilation of the forearm resistance vasculature by experimental endotoxemia is consistent with previous reports (22, 23).

The mechanism whereby BH₄ improved endothelial function cannot be derived directly from these experiments. Because BH₄ is a cofactor of NOS, BH₄ administration might increase NO formation (19). However, exogenous BH₄ had no effect on resting FBF in our study; this response renders

**Table 2.** FBF, plasma BH₄, vitamin C, and hsCRP at baseline and 205 and 320 min after LPS with coadministration of placebo, BH₄, or vitamin C and BH₄ and vitamin C in local venous effluent in the intervention arm 320 min after LPS

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>205 min After LPS</th>
<th>320 min After LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Systemic</td>
<td>Local</td>
<td></td>
</tr>
<tr>
<td>FBF, ml/min⁻¹·100 ml⁻¹</td>
<td>1.0±0.17</td>
<td>1.16±0.39</td>
<td></td>
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<tr>
<td>BH₄, nmol/l</td>
<td>15.2±9.8</td>
<td>18.8±11.8</td>
<td>14.4±7.36</td>
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<tr>
<td>Vitamin C, μmol/l</td>
<td>76.4±14.6</td>
<td>52.1±16.3*</td>
<td>43.3±12.1*</td>
</tr>
<tr>
<td>hsCRP, mg/l</td>
<td>1.58±1.03</td>
<td>1.34±0.85*</td>
<td>2.43±1.25*</td>
</tr>
<tr>
<td>FBF, ml/min⁻¹·100 ml⁻¹</td>
<td>0.99±0.22</td>
<td>1.09±0.22</td>
<td>524.6±356.9*</td>
</tr>
<tr>
<td>BH₄, nmol/l</td>
<td>15.4±6.4</td>
<td>15.7±7.2</td>
<td>50.5±9.6*</td>
</tr>
<tr>
<td>Vitamin C, μmol/l</td>
<td>67.1±19.38</td>
<td>41.7±16.8*</td>
<td>50.5±9.1*</td>
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<tr>
<td>hsCRP, mg/l</td>
<td>1.23±0.93</td>
<td>1.08±0.76</td>
<td>1.93±0.98*</td>
</tr>
<tr>
<td>FBF, ml/min⁻¹·100 ml⁻¹</td>
<td>1.08±0.34</td>
<td>1.22±0.27</td>
<td>2226.2±19039.9†</td>
</tr>
<tr>
<td>BH₄, nmol/l</td>
<td>13.3±8.7</td>
<td>17.3±5.4</td>
<td>18.8±7.9</td>
</tr>
<tr>
<td>Vitamin C, μmol/l</td>
<td>69.2±9.8</td>
<td>48.1±11.7*</td>
<td>23.6±11.9†</td>
</tr>
<tr>
<td>hsCRP, mg/l</td>
<td>0.87±0.78</td>
<td>0.76±0.51*</td>
<td>2134.8±1206.5*†</td>
</tr>
</tbody>
</table>

Values are means ± SD, (n = 8). FBF, forearm blood flow; hsCRP, high-sensitivity C-reactive protein; BH₄, tetrahydrobiopterin. At 205 min after LPS, FBF was measured 5 min after start of placebo, BH₄, or vitamin C. *P < 0.02 vs. baseline; †P < 0.02 vs. systemic concentration (Wilcoxon’s matched-pairs test).
substrate deprivation unlikely for the observed regional and systemic vasodilation. This is consistent with previous experiments showing that BH₄ did not affect endothelium-dependent or -independent vasodilation in healthy volunteers (34).

BH₄ exerts antioxidant effects that might prevent degradation of vasoactive NO by ROS (32). Nevertheless, the effect of BH₄ on ACh-induced vasodilation is not likely a nonspecific antioxidant effect alone: a clinical trial in smokers demonstrated that the antioxidant capacity of the structurally related tetrahydroneopterin (NH₄) was comparable to that of BH₄ but that NH₄ had no effect on impaired endothelium-dependent vasodilation (10).

LPS reduced circulating concentration of vitamin C; this finding is consistent with previous experiments (23). Interestingly, no effect of inflammation on forearm BH₄ plasma levels would be expected from cell culture experiments, which demonstrated a rise of BH₄ levels caused by upregulation of GTP cyclohydrolase I by combinations of proinflammatory cytokines (15, 30). Acute inflammation is reflected in our model; therefore, transcriptional effects that occur after prolonged exposure to inflammatory mediators have not been evaluated. Our finding of a vitamin C-induced increase in BH₄ concentration is compatible with animal studies showing that vitamin C can improve endothelial dysfunction by scavenging free radicals and elevating vascular BH₄ concentration (7). The increase in BH₄ after vitamin C was small compared with plasma concentration after BH₄ administration. Nevertheless, even a small increase in BH₄ could correct a relative deficiency of BH₄, which would otherwise lead to increased free radical formation. This would counteract uncoupling of oxygen reduction and arginine oxidation during suboptimal BH₄ concentration accordingly. The mechanism responsible for the increase in local BH₄ plasma concentration after vitamin C

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**Fig. 1.** ACh- and glyceryltrinitrate-induced vasodilation at baseline (○) and 3.5 h after LPS infusion (●) with coadministration of placebo. Drug effects are expressed as ratio of blood flow in the intervention arm to blood flow in the control arm; predose ratio is defined as 100%. FBF, forearm blood flow. Values are means ± SD (n = 8). *P < 0.04 vs. baseline (ANOVA).

**Fig. 2.** ACh- and glyceryltrinitrate-induced vasodilation before (○) and 3.5 h after LPS infusion (●) with coadministration of tetrahydrobiopterin (BH₄). Drug effects are expressed as described in Fig. 1. Values are means ± SD (n = 8). *P = not significant vs. baseline (ANOVA).
may be chemical stabilization of BH₄, which was described for intracellular BH₄ (12). Our cell culture experiments also confirm previous studies (12) showing a substantial increase of intracellular BH₄ in HUVEC incubated with LPS and vitamin C. It may therefore be hypothesized that intracellular BH₄ concentration is much higher and only poorly reflected by elevated plasma levels after vitamin C infusion. Although elevated intracellular BH₄ concentration could enhance NO formation, we can only speculate if this explains improved endothelium-dependent vasodilation. The direct mechanisms underlying restoration of endothelial function in vivo cannot be derived from these cell culture experiments. Inflammatory stimuli lead to a prooxidant state, which could result in decreased BH₄ levels. On the contrary, a previous study in HUVEC demonstrated that proinflammatory cytokines increase intracellular BH₄ by upregulation of cyclohydrolase I activity (30). In our experiments, intracellular BH₄ was not altered in LPS-stimulated HUVEC. This could be due to an experimental design different from that of Rosenkranz-Weiss et al. (30), who used proinflammatory cytokines, and not LPS, to stimulate HUVEC.

Our results are in good agreement with studies showing that high doses of BH₄ improve impaired endothelial function in patients with hypercholesterolemia (34), diabetes mellitus type 2 (11), and heart failure (31). In these disease states, chronic inflammation is present, as evidenced by elevated plasma hsCRP concentration (1, 8, 33). It has been demonstrated that C-reactive protein can attenuate endothelial cell NO production (38), but its vasorelaxant properties are poor (37). BH₄ or vitamin C did not influence hsCRP. Thus hsCRP is unlikely to influence endothelial dysfunction in our study. Interestingly, hsCRP levels significantly decreased 205 min and increased 320 min after LPS. This biphasic pattern of hsCRP after induction of E. coli endotoxemia is described for the first time in this experimental setting and cannot be explained. On the basis of our findings, it is unlikely that a dilution effect is responsible for the decrease of hsCRP. The systemic inflammatory response to endotoxemia as assessed by WBC, IL-6, IL-1β, IFN-γ, and MCP-1 was not influenced by BH₄ or vitamin C. This argues against a confounding influence of altered systemic humoral reactivity on endothelium-dependent vasodilation during treatment with BH₄ or vitamin C.

Our data further confirm the results of our previous study, where vitamin C restored endothelial dysfunction in the forearm vasculature after LPS (23). These findings suggest that degradation of NO by ROS can be counteracted by administration of antioxidants. However, it can also be speculated that protection of BH₄ by vitamin C against ROS and, consequently, increased intracellular BH₄ levels contributes to restoration of endothelium-dependent vasodilation. Indeed, this mechanism was demonstrated in vitro in porcine aortic endothelial cells (12).

In conclusion, we have demonstrated that BH₄ can reverse inflammation-induced impairment of endothelium-dependent vasodilation. Vitamin C exerts its beneficial effects possibly not only by its potent antioxidant capacity but also by stabilization of intracellular BH₄.
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