

Tetrahydrobiopterin restores endothelial dysfunction induced by an oral glucose challenge in healthy subjects

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Ihlemann, Nikolaj, Christian Rask-Madsen, Anders Perner, Helena Dominguez, Thomas Hermann, Lars Køber, and Christian Torp-Pedersen. Tetrahydrobiopterin restores endothelial dysfunction induced by an oral glucose challenge in healthy subjects. *Am J Physiol Heart Circ Physiol* 285: H875–H882, 2003. First published May 1, 2003; 10.1152/ajpheart.00008.2003.—An oral glucose challenge causes transient impairment of endothelial function, probably because of increased oxidative stress. During oxidative stress, endothelial nitric oxide (NO) synthase (eNOS) becomes uncoupled because of decreased bioavailability of tetrahydrobiopterin (BH₄), an essential cofactor of eNOS. Therefore, we examined whether an acute supplement of BH₄ could restore endothelial dysfunction induced by an oral glucose challenge. Healthy subjects were examined in 53 experiments. Forearm blood flow was measured by venous occlusion plethysmography. Dose-response studies were obtained during intra-arterial infusion of serotonin to elicit endothelium-dependent, NO-specific vasodilation and during sodium nitroprusside (SNP) infusion to elicit endothelium-independent vasodilation. Subjects were examined before (fasting) and 1 and 2 h after an oral glucose challenge (75 g) with serotonin ($n = 10$) and SNP ($n = 8$). On different days (6*R*)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (6*R*-BH₄; $n = 10$), the active cofactor of eNOS or its stereoisomer (6*S*)-5,6,7,8-tetrahydro-L-biopterin sulfate (6*S*-BH₄; $n = 10$), which is inactive as a cofactor, was added 10 min (500 μg/min) before and during the 1-h postchallenge serotonin dose-response study. In vitro studies showed that 6*R*-BH₄ and 6*S*-BH₄ were equipotent antioxidants. Serotonin response was reduced by $24 \pm 7\%$ (at the highest dose) at 1 h postchallenge compared with fasting ($P = 0.001$) and was restored 2 h postchallenge. The reduction was reversed by the administration of 6*R*-BH₄ but not by 6*S*-BH₄. SNP responses were slightly increased 1 and 2 h postchallenge (increased by $15 \pm 13\%$ at third dose 2 h postchallenge, $P = 0.0001$). An oral glucose challenge causes transient, NO-specific, endothelial dysfunction, which may be reversed by BH₄. Transient postprandial endothelial dysfunction may be partly explained by reduced bioavailability of BH₄ and NO.

nitric oxide; postprandial; hyperglycemia

ELEVATED LEVELS OF GLUCOSE are continuous risk factors for atherosclerosis starting in the nondiabetic range

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with only postprandial hyperglycemia to the state of diabetes with chronic hyperglycemia (19). The mechanism of hyperglycemia-induced atherosclerosis is only incompletely understood.

Dysfunction of the endothelium, including impaired endothelium-dependent vasodilation, is an early hallmark of atherosclerosis, and almost any risk factor of atherosclerosis has been found to be associated with reduced nitric oxide (NO) production/bioactivity (6). More recently, endothelial dysfunction has been shown to be a prognostic marker of cardiovascular events (30).

An oral glucose challenge has been shown to cause endothelial dysfunction in patients with Type 2 diabetes and insulin resistance (7, 17) as well as in healthy subjects (1, 32). The mechanism for this (these) effect is not clear, but increased oxidative stress, i.e., an enhanced formation of reactive oxygen species, has been proposed (23, 32). Studies of endothelial cells during hyperglycemia points to the mitochondria (21) as well as endothelial NO synthase (eNOS) (8) as the origin of reactive oxygen species formation. eNOS is a complex enzyme with the ability to produce both NO as well as superoxide anion dependent of the availability of the essential cofactor tetrahydrobiopterin (BH₄) (35). In the setting of increased oxidative stress, BH₄ might be limited in amount because of its rapid breakdown (20), resulting in uncoupling of eNOS (41) and subsequent favoring of superoxide anion production rather than NO.

It is unknown whether an oral glucose challenge affects endothelial function via a relative deficiency of BH₄. We hypothesized that the postchallenge impairment of endothelial function involved a decrease of intracellular BH₄ concentrations below a critical threshold, resulting in uncoupling of NO production and impairment of endothelial function, and that this transient condition could be prevented by supplementation of BH₄. Therefore, we examined endothelium-dependent vasodilation before and after an oral glucose challenge with and without BH₄ supplement. To control for the nonspecific antioxidative effect of BH₄, control experiments were carried out using the stereoisomer (6*S*)-5,6,7,8-tetrahydro-L-biopterin sulfate (6*S*-

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BH₄). This stereoisomer of BH₄ has negligible effects as a cofactor of eNOS (18) compared with its active counterpart (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (6R-BH₄), and preparatory *in vitro* experiments showed that 6R- and 6S-BH₄ have equipotent antioxidative effects.

METHODS

Subjects. A total of 53 experiments was performed in 39 healthy subjects. Nine subjects participated in the N^G-monomethyl-L-arginine (L-NMMA)-control protocol and 5 participated in the repeated-serotonin protocol. In the four "main" protocols (see *Infusion protocols*) 25 subjects participated: 2 subjects agreed to participate in all 4 protocols, 1 participated in 3 protocols, 5 participated in 2 protocols, and 17 participated in 1 protocol. All were nonsmokers, with no history of diabetes or cardiovascular disease, and free of medication. Before participating, the subjects were screened for hypercholesterolemia and diabetes by measuring their fasting values of cholesterol and whole blood glucose. A value <6 mmol/l in total cholesterol and <5.5 mmol/l in whole blood glucose were accepted criteria for participating. The study was approved by the local ethical committee as well as the Danish Medicine Agency. After reviewing oral as well as written information, the subjects approved to participate by written consent.

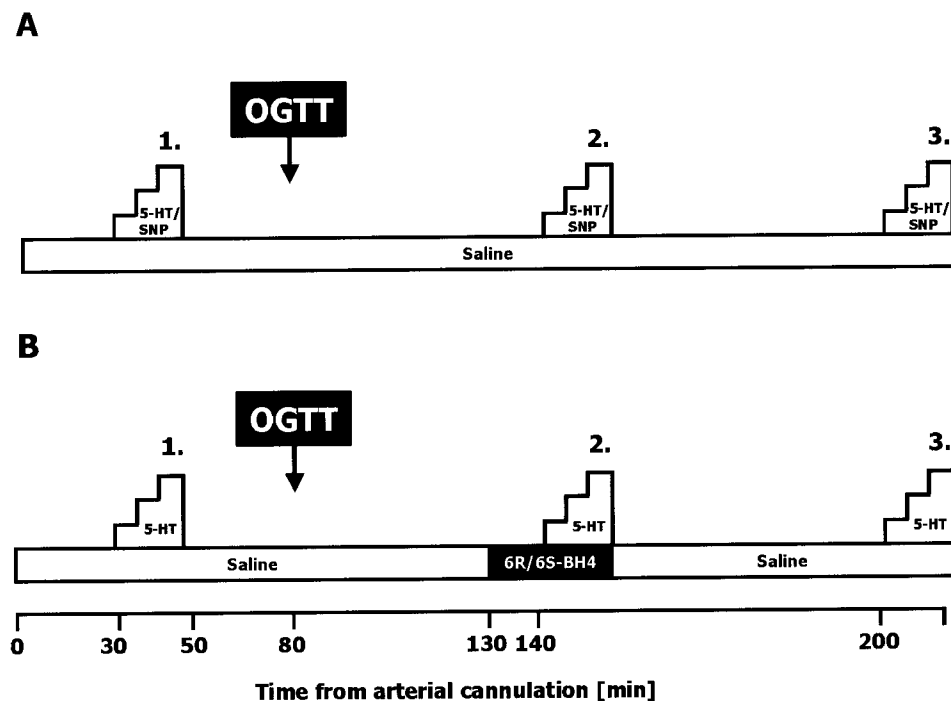
Drug preparation. BH₄ was purchased from Schircks Laboratories (Jona, Switzerland). 6R-BH₄ was purchased in stocks of 100 mg and divided into aliquots. 6S-BH₄, which is only approved for laboratory use, was purified by sterile filtration before being divided into aliquots for use on the study day. The aliquots were frozen immediately and kept at -20°C until the day of study. Serotonin and sodium nitroprusside (SNP) were diluted just before use on the day of the study. All solutions were protected from light.

***In vitro* study of antioxidative effect of BH₄.** BH₄ may have direct antioxidant effects, probably by scavenging superoxide

(38). To validate whether the 6S stereoisomer of BH₄ was suitable as a control in the human *in vivo* study, a preparatory study of the relative antioxidant effect of the two isoforms was performed *in vitro*. Xanthine oxidase was dissolved in Krebs-Ringer buffer (final concentration 2 mU/ml), and luminol (50 mM) and superoxide generation were started by the addition of xanthine (400 nM), measured at 1-min intervals. The present results have been corrected for background chemiluminescence by subtracting measured values from an experiment where xanthine was omitted. The experiment was repeated with prior addition to the buffer of either 6R-BH₄, 6S-BH₄ (concentrations as indicated), or superoxide dismutase (200 U/ml).

Vascular study. The experiments were performed in a temperature-controlled room (21–23°C) starting at 8:00 AM with the subjects in the fasting state. Subjects were in a supine position with both forearms resting slightly above the level of the heart. After local anesthesia was administered, a 20-gauge arterial cannula (Becton Dickinson; Swindon, UK) was inserted into the brachial artery of the nondominant arm ("infused arm") for infusion of drugs. A venous catheter was placed in the cubital vein of both the infused arm and the contralateral arm ("control arm") for blood sampling. In the infused arm, the catheter was placed retrogradely in a deep vein to collect blood draining from the muscle rather than the skin. Saline was infused through the arterial cannula throughout the day and interrupted by drug infusions as shown in the infusion protocols (Fig. 1). The infusion volume was kept constant at 1 ml/min by adjusting the saline infusion. Forearm blood flow was measured by using bilateral venous occlusion plethysmography (D. E. Hokanson, Bellevue, WA). The hand circulation was excluded before measurements of blood flow by inflating pneumatic wrist cuffs to 200 mmHg. Blood flow was calculated as the mean value of at least four stable readings on the plethysmograph. The analysis of flow data of the treatment was performed in a blinded fashion. Baseline blood flow was measured 30 min after cannulation, and dose-response studies were separated by 30

Fig. 1. Infusion protocols. **A:** on different days, forearm blood flow was measured during dose-response studies (*studies 1, 2, and 3*) of serotonin (5-HT) and sodium nitroprusside (SNP) fasting, and 1 and 2 h after an oral glucose tolerance test (OGTT) (5-HT-saline protocol and SNP-saline protocol). **B:** on two other days the 5-HT response was reexamined during infusion of either 6R- or 6S-BH₄ (500 µg/min) 10 min before and continued throughout the 1-h postchallenge serotonin dose-response study (5-HT-6R-BH₄ protocol and 5-HT-6S-BH₄ protocol).



min. Endothelium-dependent vasodilation was elicited by infusion of serotonin (Clinalfa; Laufwigen, Switzerland) in three incremental doses (7, 21, and 70 ng/min), and endothelium-independent vasodilation was elicited by the infusion of SNP (Nitropress, Abbott; North Chicago, IL) in three doses (0.5, 1.5, and 5 $\mu\text{g}/\text{min}$). During the dose-response studies, serotonin and SNP were infused for 5 min at each dose to ensure steady state. The doses of serotonin used are shown to cause vasodilation in healthy subjects and are equivalent to the doses used by other groups (9, 13, 29, 34, 36). The doses of SNP were chosen from previous experience in our laboratory to result in comparable levels of vasodilation.

Blood pressure and heart rate were monitored throughout the experimental day from a three-lead ECG and a transducer connected to the arterial cannula, respectively.

Infusion protocols. Six different infusion protocols (two basic control protocols and four "main" protocols) were carried out on different study days. The basic control protocols included a L-NMMA-control protocol and a repeated-serotonin protocol. In the L-NMMA-control protocol the degree of serotonin-induced vasodilation mediated by NO was examined during L-NMMA blockade to explore previous observations by Stroes et al. (29). L-NMMA (3.3 mg/min, Clinalfa; Laufwigen, Switzerland) was infused 10 min before and continued throughout a serotonin dose-response study. In the repeated-serotonin protocol the effect of repeated serotonin dose-response studies was explored. Two serotonin (also known 5-HT) dose responses were repeated with a 90-min interval as shown in the first part of Fig. 1A (*dose responses 1 and 2*) but without any other intervention. The four "main" protocols are shown in Fig. 1. In the serotonin-saline protocol, the first serotonin dose-response study was recorded in the fasting state. A 30-min pause followed to allow for blood flow to return to resting levels. The subject then received an oral glucose challenge of 75 g of glucose (180 ml of a mixture of 2.085 mmol/ml). The second and third serotonin dose-response studies were repeated 1 and 2 h after the glucose challenge (Fig. 1A). In the SNP-saline protocol, on another day, serotonin was substituted with SNP (Fig. 1A). The serotonin-6R-BH₄ protocol was similar to the serotonin-saline protocol except that 6R-BH₄ (500 $\mu\text{g}/\text{min}$) was infused 10 min before and continued throughout the second serotonin dose-response study (Fig. 1B). The serotonin-6S-BH₄ protocol was similar to the serotonin-saline protocol except that 6S-BH₄ (500 $\mu\text{g}/\text{min}$) was infused 10 min before and continued throughout the second serotonin dose-response study (Fig. 1B). Subjects participating in more than one protocol did so in a randomized fashion, and each protocol was performed at least 1 wk apart to eliminate any carry-over effect. Because all previous studies that we are aware of have shown that BH₄ does not affect smooth muscle reactivity (13, 14, 28, 31, 33, 37), we did not also perform this control experiment.

Metabolic study. Because BH₄ has been shown to affect markers of insulin resistance (26), we measured forearm glucose uptake in all protocols. Immediately before all the dose-response studies as well as before and after BH₄ infusions (Fig. 1), blood samples were obtained from arterial and venous catheters, followed by measurement of blood flow. Forearm glucose uptake was calculated as the arteriovenous difference multiplied by forearm blood flow, as described previously (42).

Biochemical analysis. Plasma glucose concentration was determined by enzymatic colorimetry, and serum insulin was determined by a microparticle enzyme immunoassay (AxSym Insulin B2D010; Abbott).

Statistical analysis. Data are given as means \pm SE. Forearm blood flow is expressed as milliliter per 100 ml of tissue per minute of the infused arm. Clinical characteristics and levels of glucose and insulin were compared by a nonpaired *t*-test. Dose-response studies were compared by using analysis of variance, mixed models (SAS Statistical Software Version 8.02, SAS Institute; Cary, NC). A major assumption is homogeneity of variance/normal distribution of residuals. This was studied in a plot of residuals versus expected values. Such graphs were satisfactory when flow was subjected to logarithmic transformation. Subjects entered the model as random effect as did the interaction between subject and dose of vasodilator. Dose of vasodilator, time (fasting, 1-h and 2-h postchallenge), and experimental protocol all entered as class effects, and the interaction of vasodilator dose and time was included in the model. Comparisons of dose-response studies were done within the study day as well as between different protocols. All comparisons were also done using forearm blood flow expressed as relative flow equaling "infused arm" per "control arm." If the results of the statistical analysis differed between the two ways to interpret blood flow, then this is mentioned in RESULTS. A *P* value < 0.05 was accepted as significant.

RESULTS

Antioxidant effect of 6R-BH₄ and 6S-BH₄. The reaction between xanthine and xanthine oxidase generated luminol-enhanced chemiluminescence ($7,537 \pm 224$ counts/s at 1 min), which was reduced by time ($1,425 \pm 159$ at 4 min, $P < 0.001$). The values of chemiluminescence were similarly affected by 6R-BH₄ and 6S-BH₄ in the dose range examined (at 1 μM , $5,427 \pm 137$ vs. $5,580 \pm 92$ counts/s; at 2.5 μM , $2,812 \pm 121$ vs. $2,405 \pm 182$ counts/s; and at 5 μM , $1,151 \pm 57$ vs. $1,451 \pm 59$ counts/s at 1 min). The effects of 6R-BH₄ and 6S-BH₄ were unaffected by time (data not shown). In contrast, SOD and 100 μM 6R-BH₄ or 6S-BH₄ totally abolished the chemiluminescence at all time points.

Subjects and response to the glucose challenge. The clinical characteristics of the subjects participating in the four "main" protocols are shown in Table 1. The subjects participating in each protocol were comparable according to the parameters listed. Characteristics of subjects participating in the two control protocols were comparable (data not shown). Table 2 shows the results of glucose and insulin before and after the oral glucose challenge. The responses are comparable to those observed in other studies (17, 32). The levels of glucose and insulin were not different in the serotonin-saline protocol compared with the other protocols.

Forearm glucose uptake increased from 0.7 ± 0.2 $\mu\text{mol} \cdot 100$ ml tissue⁻¹·min⁻¹ in the fasting state to 3.0 ± 0.6 and 1.4 ± 0.2 $\mu\text{mol} \cdot 100$ ml tissue⁻¹·min⁻¹ 1 and 2 h postchallenge, respectively. Glucose uptake was not affected by infusion of 6R-BH₄ or 6S-BH₄ (data not shown).

Forearm blood flow. Resting blood flow was unaffected by the glucose challenge in all protocols except the serotonin-saline protocol where a minor reduction was present (2.2 ± 0.2 vs. 1.9 ± 0.1 ml·100 ml tissue⁻¹·min⁻¹, $P = 0.01$). Infusion of 6R-BH₄ or 6S-BH₄ did not change resting blood flow (2.7 ± 0.3 vs.

Table 1. Characteristics of the study population participating in each of the four "main" protocols

Protocol	Serotonin + Saline	Serotonin + 6R-BH ₄	Serotonin + 6S-BH ₄	SNP + Saline
<i>n</i>	10	10	10	8
Sex (M/F)	6/4	6/4	6/4	5/3
Age, yr	53.0 ± 2.2	53.4 ± 2.0	52.5 ± 2.2	52.4 ± 3.6
BMI	22.7 ± 0.6	23.3 ± 0.4	23.8 ± 0.6	23.1 ± 1.2
Forearm volume, ml	980 ± 160	983 ± 57	995 ± 52	975 ± 83
HbA _{1c} , %	5.2 ± 0.1	5.2 ± 0.04	5.1 ± 0.1	5.2 ± 0.1
Systolic BP, mmHg	144 ± 6	134 ± 5	131 ± 4	138 ± 6
Diastolic BP, mmHg	75 ± 4	72 ± 3	72 ± 3	72 ± 4
Triglyceride mmol/l	0.8 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.2
Total cholesterol, mmol/l	5.2 ± 0.2	5.0 ± 0.2	4.7 ± 0.2	4.6 ± 0.4
Cholesterol				
HDL, mmol/l	1.7 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
LDL, mmol/l	3.2 ± 0.2	2.8 ± 0.2	2.8 ± 0.2	2.5 ± 0.4
VLDL, mmol/l	0.3 ± 0.03	0.4 ± 0.2	0.3 ± 0.03	0.4 ± 0.1

Values are means ± SE; *n*, number of participants. BH₄, tetrahydrobiopterin; SNP, sodium nitroprusside; BMI, body mass index; HbA_{1c}, glycated hemoglobin; BP, blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

2.8 ± 0.3 ml·100 ml tissue⁻¹·min⁻¹ and 1.9 ± 0.2 vs. 2.0 ± 0.2 ml·100 ml tissue⁻¹·min⁻¹ before and after 6R- and 6S-BH₄, respectively).

In the L-NMMA-control protocol, infusion of L-NMMA completely blocked the serotonin-induced vasodilation (2.0 ± 0.1, 2.0 ± 0.1, and 2.0 ± 0.2 ml·100 ml tissue⁻¹·min⁻¹ at the three serotonin doses), confirming that serotonin-induced vasodilation is NO mediated. The repeated-serotonin protocol showed a minor but insignificant increase during the second serotonin response (2.5 ± 0.4, 3.4 ± 0.4, and 4.7 ± 0.9 ml·100 ml tissue⁻¹·min⁻¹ and 3.2 ± 1.1, 4.1 ± 0.9, 5.6 ± 1.6 ml·100 ml tissue⁻¹·min⁻¹) at the three serotonin doses during the first and second serotonin dose response, respectively (*P* = 0.12).

In the serotonin-saline protocol (Fig. 2A), serotonin infusion resulted in a dose-dependent increase in forearm blood flow during fasting, which was significantly blunted 1 h postchallenge (*P* = 0.0012), a reduction of 24 ± 7% at dose 3. Two hours postchallenge, the serotonin response was still lower than during fasting conditions (reduced by 5 ± 10% at dose 3) but not statistically different from fasting (*P* = 0.17).

The SNP response was augmented after the glucose challenge (Fig. 2B). One hour postchallenge, a minor but significant augmentation was seen (*P* = 0.004), and at 2 h postchallenge this augmentation was more obvious (increased by 15 ± 13% at dose 3, *P* = 0.0001 considering all doses).

During infusion of 6R-BH₄, the serotonin dose-response curve 1 h postchallenge was not different from that obtained during fasting (*P* = 0.36) and neither was the 2-h postchallenge response (*P* = 0.31) (Fig. 3A). However, during infusion of 6S-BH₄ (Fig. 3B), the serotonin dose-response curves were significantly impaired both 1 (*P* < 0.0001) and 2 h postchallenge (*P* = 0.034). When the 1-h postchallenge serotonin response in the serotonin-6R-BH₄ protocol was compared with the serotonin-6S-BH₄ protocol, there was a significant difference (*P* < 0.001) documenting that the two experiments provided different results. The fasting serotonin dose-response study in the serotonin-6S-BH₄ protocol did not differ from that obtained in the serotonin-saline protocol (*P* = 0.57).

Because of the minor differences in forearm blood flow before the serotonin dose-response study in the three protocols, serotonin-saline, serotonin-6R-BH₄, and serotonin-6S-BH₄ flow data were also analyzed by using delta forearm blood flow (stimulated flow - baseline flow) as used previously (39). Figure 4 summarizes the effects of the infusions of saline, 6R-BH₄, or 6S-BH₄ on the 1-h postchallenge serotonin response. After baseline correction, there was still a significant increased serotonin response during the 6R-BH₄ infusion compared with the saline (*P* < 0.05) 1 h postchallenge. Although reduced, the serotonin response during 6S-BH₄ was only borderline statistical significant (*P* = 0.05) compared with saline.

Table 2. Glucose and insulin levels before and after an oral glucose challenge in each study protocol

Protocol	Serotonin + Saline	Serotonin + 6R-BH ₄	Serotonin + 6S-BH ₄	SNP + Saline
P-glucose, mmol/l				
Fasting	5.2 ± 0.1	5.4 ± 0.1	5.6 ± 0.1	5.2 ± 0.2
1-h postchallenge	8.4 ± 0.6	9.9 ± 0.7	9.3 ± 0.6	8.6 ± 0.7
2-h postchallenge	7.4 ± 0.5	7.4 ± 0.6	7.6 ± 0.4	7.0 ± 0.5
S-insulin, μU/ml				
Fasting	4.3 ± 0.5	4.8 ± 0.5	5.9 ± 1.0	4.1 ± 0.8
1-h postchallenge	40.2 ± 5.0	42.8 ± 5.0	54.4 ± 9.1	44.4 ± 9.7
2-h postchallenge	32.3 ± 7.1	34.0 ± 7.8	35.0 ± 8.5	33.5 ± 9.3

Values are means ± SE. P, plasma; S, serum.

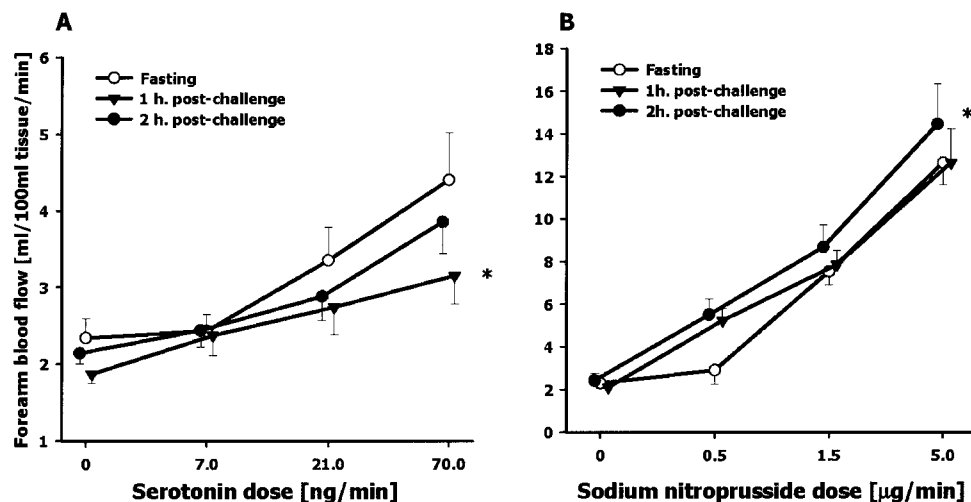


Fig. 2. Effect of an oral glucose challenge on endothelium-dependent and -independent vasodilation. Dose-response studies were carried out at fasting, 1 h postchallenge, and 2 h postchallenge. Serotonin response was significantly impaired 1 h postchallenge, $*P = 0.0012$, and was partly restored 2 h postchallenge, $*P = 0.17$ (A). As opposed to this, SNP responses were slightly augmented 1 h postchallenge, $P = 0.004$ and more so 2 h postchallenge, $*P = 0.0001$ (B). Data are presented as means \pm SE.

Systemic circulatory response. Blood pressure and pulse rate were not affected by the vasoactive infusions and did not change systematically throughout the experiment day or between study protocols. Blood flow was also not affected in the “control arm” during drug infusions (data not shown).

DISCUSSION

The present study shows that BH₄ restores endothelium-dependent vasodilation induced by an oral glucose challenge in the forearm of healthy subjects. The effect can be attributed to an effect of BH₄ as a cofactor of eNOS rather than merely a direct antioxidant effect, because we do not observe this protective effect during infusion of 6S-BH₄. The impaired endothelium-dependent vasodilation observed postchallenge can be interpreted as a reduction in NO production/bioactivity, because serotonin-induced vasodilation is completely blocked by L-NMMA. These results suggest that insufficient BH₄ concentrations may be a limiting factor for endothelial function, even in healthy people during a normal physiological situation.

Previous studies have shown that a glucose challenge impairs flow-mediated vasodilation of a conduit (brachial) artery in healthy subjects (1, 32) and even more so in patients with impaired glucose tolerance or diabetes (7, 17). Our results extend this knowledge to include resistance vessels of the forearm by showing a significant impairment of endothelial-dependent vasodilation 1 h postchallenge as measured by plethysmography (4). We observe a minor change in basal flow in the serotonin-saline protocol postchallenge. It seems unlikely that this change in basal flow is the cause of impaired endothelium-dependent vasodilation postchallenge because impairment of endothelium-dependent vasodilation postchallenge is readily reproduced in the serotonin-6S-BH₄ protocol where basal flow is unchanged.

It is largely unknown what causes endothelial dysfunction after an oral glucose challenge. Two main features potentially modulate endothelial function postchallenge: the rise in blood glucose and the concomitant rise in insulin. Hyperinsulinemia has a well-known ability to cause augmentation of endothelium-

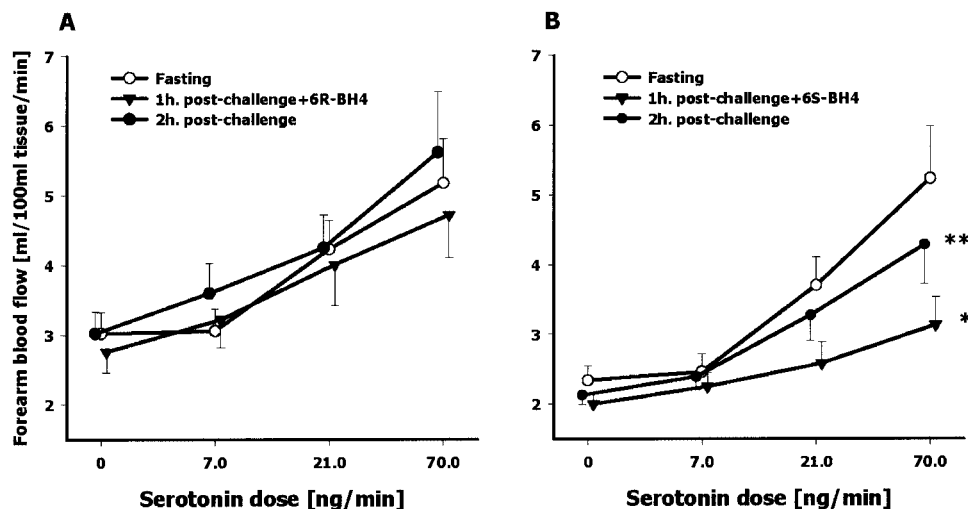


Fig. 3. Effect of an oral glucose challenge on endothelium-dependent vasodilation during infusion of BH₄, 1 h postchallenge, and 2 h postchallenge. Dose-response studies were carried out at fasting, 1 h postchallenge, and 2 h postchallenge. When 6R-BH₄ was infused 10 min before and during the 1-h postchallenge dose-response study, serotonin responses were not different from that obtained at fasting, neither 1-h ($P = 0.36$) nor 2-h ($P = 0.31$) postchallenge (A). 6S-BH₄ infusion was not able to counteract impairment of the serotonin response neither 1-h ($*P < 0.0001$) nor 2-h ($**P = 0.034$) postchallenge (B). Data are presented as means \pm SE.

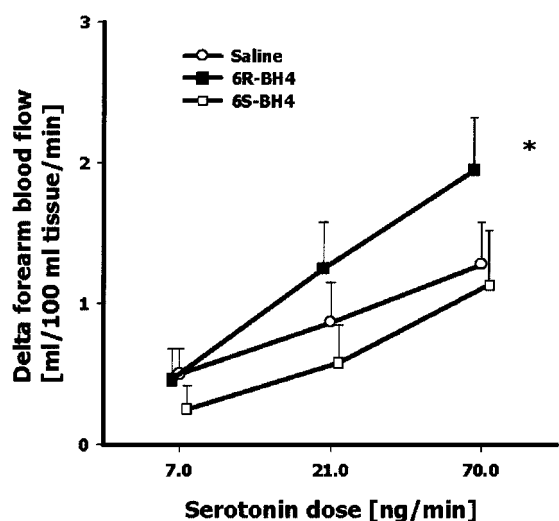


Fig. 4. Delta forearm blood flow 1-h postchallenge during saline infusion, 6R-BH₄ infusion, or 6S-BH₄. After corrections for baseline changes, the serotonin response was still increased during 6R-BH₄-infusion compared with saline (**P* < 0.05).

dependent vasodilation (27) in healthy subjects. In endothelial cells, the mechanism has been shown to include an activation of eNOS via the intracellular insulin signal transduction pathway (15). Hyperglycemia, on the other hand, may decrease NO activity by several mechanisms. Glucose has the ability to quench NO directly (5), and hyperglycemia causes increased superoxide anion production (8) and subsequent scavenging of NO during the formation of the peroxynitrite radical (11). Several studies have shown that hyperglycemia-induced endothelial dysfunction can be corrected during antioxidant therapy both in vitro (10) and in humans during experimental hyperglycemia (3) as well after an oral glucose challenge (32). Taken together, the observed impairment of endothelium-dependent vasodilation postchallenge is most likely mediated by hyperglycemia-induced oxidative stress (17, 23) despite possible eNOS activation during hyperinsulinemia.

We find that endothelium-independent vasodilation is slightly augmented postchallenge in contrast to a previous study (17) where endothelium-independent vasodilation was determined. Postprandial hyperinsulinemia could be the mediator of an augmented response to a NO donor. In vitro studies of smooth muscle cells conducted by Kahn et al. (16) has shown that insulin causes increased NO sensitivity. In accordance with this, a previous study (24) has shown that the vasodilatory response to SNP was augmented during hyperinsulinemia. Because acute hyperglycemia does not seem to affect endothelium-independent vasodilation (39), we suggest that postchallenge hyperinsulinemia is the mediator of the augmented postchallenge SNP response. If so, the impairment of endothelium-dependent vasodilation postchallenge may have been underestimated.

The main finding of the present study is that an acute supply of 6R-BH₄ counteracts the impairment of endothelium-dependent vasodilation induced by an oral glucose challenge. Conditions of oxidative stress are known to reduce NO activity through the scavenging of NO by superoxide anion during the formation of peroxynitrite. Moreover, recent evidence also indicates that peroxynitrite is very potent in oxidizing and hence inactivating BH₄ (20) as well as reducing the binding ability of the cofactor to the heme domain of eNOS (22). Thereby a vicious cycle can propagate: superoxide anion scavenges NO resulting in the formation of peroxynitrite, which in turn, reduces BH₄ availability causing uncoupling of eNOS; eNOS uncoupling results in further reduction of NO formation because superoxide anion rather than NO production is favored at this state of the enzyme (35). We find that infusion of 6R-BH₄ restores both the serotonin response during which it is infused (1 h postchallenge) but also restores the serotonin response 2 h postchallenge. This longer-lasting effect of supplying 6R-BH₄ could be explained by the effect of BH₄ to prevent a vicious cycle from propagating.

BH₄ may have direct antioxidative properties unrelated to its effect as a cofactor of eNOS, which might affect endothelium-dependent vasodilation. To test this hypothesis, we infused 6S-BH₄, a pteridine with a binding affinity for eNOS that is at least 100-times lower than that of 6R-BH₄ (18) but with similar antioxidative effect (as shown in the in vitro study). 6S-BH₄ did not counteract the impairment of endothelium-dependent vasodilation as observed with 6R-BH₄, the active cofactor. The response during the serotonin-6S-BH₄ protocol was very similar to that observed in the serotonin-saline protocol, which indicates that the effect of 6R-BH₄ can be attributed to its effect as a cofactor of eNOS. Also, because BH₄ infusion did not change forearm glucose uptake, the effect of BH₄ on endothelium-dependent vasodilation is independent of forearm glucose uptake, which might relate to endothelial function (2).

Previous human studies are in accordance with the concept that supplement of BH₄ can improve endothelial function in disease states characterized by oxidative stress (12–14, 25). However, in healthy subjects BH₄ supplementation has not previously been shown to affect endothelial function, indicating that the cofactor does not limit eNOS activity in healthy conditions (13, 14). Here we show that a glucose challenge causing an increase in plasma glucose similar to that seen after a typical meal (40), causes an apparent deficiency of BH₄, and that a total of 25 min of BH₄ administration prevents the endothelial dysfunction seen up to 2 h after glucose ingestion. These results provide an increased understanding of the mechanisms of dynamic endothelial dysfunction in healthy people. Future studies should determine whether BH₄ deficiency is even more pronounced in the postprandial hyperglycemia seen in impaired glucose tolerance and

diabetes, and itself is a risk factor for the development atherosclerosis.

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