Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 516-523

TETRAHYDROBIOPTERIN AS ANOTHER EDRF IN MAN

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Received October 5, 1994

Endotoxin and inflammatory cytokines downregulate expression of constitutive nitric oxide synthase (cNOS) in human vascular endothelial cells with concomitant increase of tetrahydrobiopterin synthesis in these cells and parallel upregulation of inducible NOS expression in smooth muscle cells, indicating compartmentalized nitric oxide (NO) production under septic conditions in man. In this report the compartmentalization has been further studied using dual chamber cell cultures with inflammatory activated human endothelial cells. We show that endothelial cells secrete BH_4 vectorially into the basal direction thereby providing underlining smooth muscle cells with the cofactor necessary for NO production. Furthermore, by laser Doppler velocimetry we show that intraarterial infusion of BH_4 induces strong vasodilatation in man. Consumption of L-arginine and production of cyclic GMP increased and therefore imply NO as second messenger. Thus the discovery of an endothelium-derived factor regulating NOS activity would reconcile the concept of an inflammatory EDRF that is not NO itself but results in NO-dependent vasodilatation in man.

Control of vascular tone including vasodilatation depends on secretory endothelial cell products (1). In animal bioassay models the endothelium-derived relaxing factor (EDRF) released upon stimulation with acetylcholine (2) has been identified as nitric oxide (NO) (3) which is synthesized by the endothelial cells in small amounts under physiologic conditions (4). NO activates soluble guanylate cyclase, and the resulting smooth muscle relaxation is mediated by cyclic GMP (5-7). Nitric oxide synthase (NOS) enzymes responsible for the bioynthesis of NO from L-arginine are expressed in the vasculature in two forms, a constitutive (cNOS) and an inducible (iNOS) form, both isoforms requiring tetrahydrobiopterin (BH_4) as cofactor (8). BH_4 synthesis in human endothelial cells is highly regulated by inflammatory signals or deactivating cytokines (9), and in these cells intracellular BH_4 closely correlates with intracellular cyclic GMP levels (9,10). Yet BH_4 synthesis appears not to serve only endothelial cell needs as most of the synthesized BH_4 is released (9). Furthermore, it has been found in

0006-291X/94 $5.00

human vascular cells that endotoxin and inflammatory cytokines upregulate iNOS expression in smooth muscle cells while cNOS expression is downregulated in endothelial cells (11), indicating a compartmentalized NO production under septic conditions. We therefore investigated whether endothelial BH_4 is secreted in a directed manner providing underlining vascular smooth muscle cells with cofactor for NO production, and if so, whether BH_4 has vasodilating properties in man.

MATERIALS AND METHODS

Cell cultures: Co-cultures and dual chamber cultures. Smooth muscle cells isolated from rat aorta (12) were seeded into 6-well cluster plates and human endothelium-derived EA.hy 926 cells (13,14) were seeded into Cyclopore™ 0.45μm membrane tissue culture inserts (Falcon Becton Dickinson Labware, Lincoln Park, NJ, USA). Both cell types were grown separately to confluence in IMDM (Gibco Europe, Basel, Switzerland) supplemented with 10% fetal calf serum (Gibco) and 50μg/ml gentamycin, referred to as complete IMDM. Confluent endothelial cells on membranes were preincubated for 24h in fresh complete IMDM with or without 1μg/ml LPS, then washed with PBS, and placed into dishes containing confluent cultures of smooth muscle cells in fresh complete IMDM. Co-cultures were incubated for 24h at which time medium was removed from the lower compartments for analysis of nitrite by the Griess method as described previously (15). To study vectorial secretion of BH_4, confluent monolayers of endothelial cells on membrane filters (16) were activated with 1μg/ml LPS and 100U/ml IFNγ for 48h, then washed and placed into fresh culture plates containing 1ml prewarmed (37°C) complete IMDM in the lower compartment (basal). 1ml of warmed complete IMDM containing 20 nmol/l neopterin was added to the upper compartment (apical). The dual chamber system was further incubated at 37°C, 5% CO_2. At the times indicated, samples were removed from both compartments, oxidized and analyzed for pterins as described (15).

Laser-Doppler velocimetry. 6R-Tetrahydrobiopterin (Schircks Laboratories, Jona, Switzerland) was dissolved immediately before use in 0.9% NaCl and infused through a Teflon catheter inserted into the left Arteria brachialis at a constant rate of 1ml/min using an automatic perfusor system. The response of the vasculature in the musculature and in the skin was measured with a single fiber Perimed II flow probe (Perimed, Stockholm, Sweden) inserted into the Musculus brachioradialis, and with a flow probe in a flat guide taped to the skin. During the experiment, test persons were recumbent at a room temperature of 24°C. L-NMMA (N^G-monomethyl-L-arginine, Calbiochem corp., La Jolla, CA) and L-arginine for parenteral use (Laboratorien Hausmann AG, St. Gallen, Switzerland) were diluted in 0.9% NaCl in a second perfusor syringe and infused at the indicated doses along with BH_4.

Metabolite measurements. cGMP was quantified by EIA (Biotrak cGMP, Amersham International plc, Amersham UK) according to the manufacturer's instructions. The assay sensitivity was 46 fmol/well. Means ± SD from quadruplicate measurements, $p < 0.001$ for each experiment. L-arginine and BH_4, detected as biopterin after acidic oxidation, were measured as described previously (15).

RESULTS AND DISCUSSION

Vectorial secretion of BH_4 by human vascular endothelial cells. In the present study we have observed that the release of BH_4 synthesized by endothelial cells activated with lipopolysaccharide (LPS) and interferon-gamma (IFNγ) was not random, but directed and that BH_4 was secreted basolaterally, i.e. into the direction of the smooth muscle layers of blood

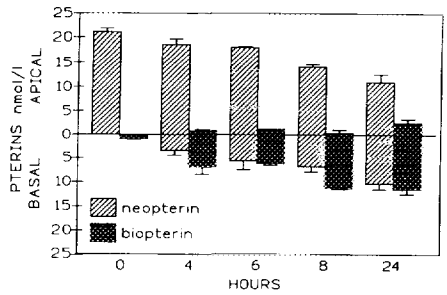


FIGURE 1.
Basolateral secretion of BH_4 by human endothelial cells. Human vascular endothelium-derived EA.hy 926 cells were cultured on porous membranes forming an upper (apical) and lower (basolateral) compartment in 6-well tissue culture plates and primed to synthesize BH_4. Neopterin, a pterin synthesized in only scant amounts by endothelial cells, was added to the upper (apical) compartment as tracer to follow diffusion of pterins through the cell monolayer from one compartment to the other. Means ± SD from triplicate wells, data representative for one out of three separate experiments.

vessels (Fig.1). Endothelial cells were grown on membrane inserts and primed to secrete BH_4 with 1μg/ml LPS, an endotoxin dose not able to induce measurable NO secretion in vascular smooth muscle cells (17) or in the human endothelial cells themselves (Table 1). When these inserts were transfered and cocultured with smooth muscle cells, NO synthesis was induced in the same by the primed endothelial cells (Table1). BH_4 is well known to be a limiting factor for NO synthesis in smooth muscle cells, and exogenously added BH_4 more than doubles NO

Table 1. NO production by underlining vascular smooth muscle cells cocultured with human endothelial cells grown in filter inserts and primed to secrete BH_4

CELL TYPE	LPS 1μg/ml	BH_4 pmol/10^6 cells*	NITRITE μmol/10^6 cells*
Endothelium	no	9.6 ± 1.4	< 0.1
Endothelium	yes	37.1 ± 1.9	< 0.1
Smooth muscle	no	< 0.08	< 0.1
Smooth muscle	yes	< 0.08	< 0.1
Co-culture endothelium-smooth muscle	no	n.d.	3.6 ± 2.1[$]
Co-culture endothelium-smooth muscle	yes	n.d.	9.3 ± 3.8[$&]

*Mean ± SE from triplicate wells and duplicate measurements. [$]Compared to control $p < 0.01$. [&]Compared to smooth muscle cells cocultured with endothelial cells not stimulated with lipopolysaccharide (LPS) $p < 0.05$. n.d., not determined.

production even in fully activated cells (17). Taken together these findings suggest that BH_4 could serve as an endothelium-derived factor mediating NO dependent smooth muscle relaxation especially under septic conditions when smooth muscle cell iNOS is highly induced. Under these circumstances endothelium-derived BH_4 would act as a first messenger that triggers NO production in the smooth muscle cells, that in turn activates synthesis of cGMP, mediating relaxation as a third messenger. Thus, besides NO as physiologic EDRF, BH_4 would act as another EDRF under septic conditions in man.

Vasodilating properties of BH_4 in man. A profound and long-lasting vasodilatation of isolated rat aorta in response to BH_4 has recently been described (18). To further elucidate the potential role of BH_4 as an EDRF we turned to human experimentation. Because BH_4, a natural intermediate of human metabolism has no known toxicity in doses up to 20mg/kg of body weight (19,20), we thought it save to perform self experiments with intraarterial infusions of BH_4 in two of us (AS and MS). BH_4 infused into the left Arteria brachialis induced a prompt, and marked local vasodilatation in a dose dependent manner in both individuals (Figs. 2 and 3) without affecting blood flow elsewhere, and without causing changes in blood pressure or pulse rate. The vascular response was much more intense in skeletal muscle than in the skin (Figs. 3A and B). But it cannot be inferred that skin vasculature is less responsive to BH_4 because skin blood flow and surface temperature dropped in the nonperfused limb during both experiments (Figs. 3B and C). This was attributed to a concomitant constrictive cutaneous vasomotor reaction due to the experimental conditions with a lightly covered recumbent test person in a room with an ambient temperature of 24°C.

After having established that BH_4 has strong vasodilating properties in man, we sought to relate BH_4 mediated vasodilatation to the endogenous nitrovasodilator system. We therefore

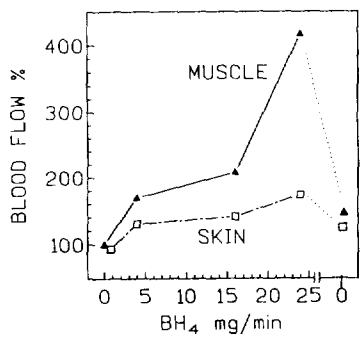
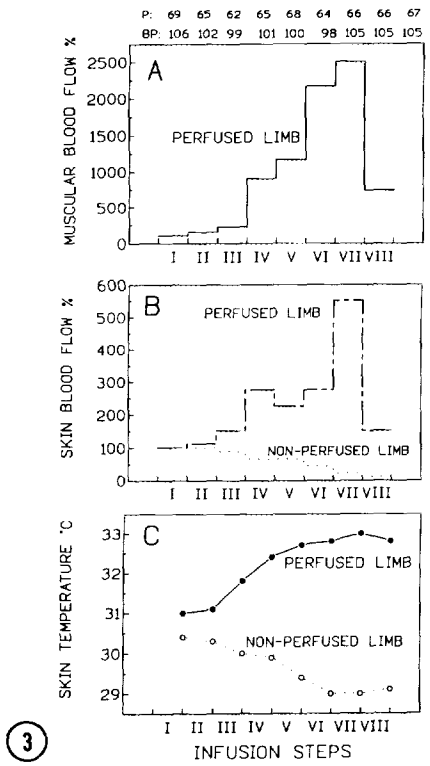


FIGURE 2.
Local hemodynamic response to BH_4 in skeletal muscle and skin. Graded concentrations of BH_4 were infused into the Arteria brachialis in 0.9 % NaCl at a rate of 1ml/min. Distal blood flow in the same arm was continuously measured by laser-Doppler velocimetry (25-27). Changes were calculated from arbitrary flow units integrated over the last 5 minutes of each 15 minute dosing step. Blood pressure and pulse rate were monitored every 15 minutes by an automatic device and remained unchanged during the experiment.



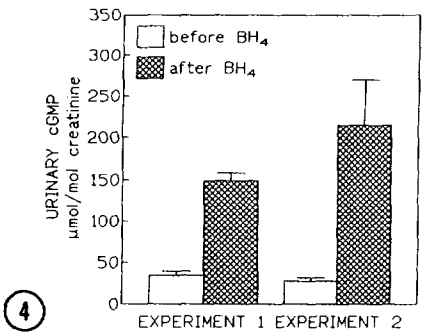


FIGURE 3.

Hemodynamic responses to infusion of graded doses of BH_4, BH_4 plus L-NMMA, or BH_4 plus graded doses of L-arginine into the Arteria brachialis. Blood flow in the muscle (**A**) and the skin (**B**) were continuously recorded by laser Doppler velocimetry using flow probes as described in Fig. 2. (**C**) Skin temperature. Mean arterial blood pressure (BP), and pulse rate (P), are shown on top of the figure. A protocol with following infusion steps each lasting 15 minutes was applied: **I**, 0.9 % NaCl; **II**, BH_4, 8 mg/min; **III**, BH_4, 16 mg/min; **IV**, BH_4, 32 mg/min; **V**, BH_4, 16mg/min plus L-NMMA, 4µmol/min; **VI**, BH_4, 16 mg/min plus L-arginine, 40 µmol/min; **VII**, BH_4, 16 mg/min plus L-arginine, 80 µmol/min; **VIII**, 0.9 % NaCl. The infusion rate was 1ml/min constantly. Changes were calculated from arbitrary flow units integrated over 5 minutes following equilibration for 10 minutes at each infusion step.

FIGURE 4.

Cyclic GMP concentrations in urine before and after intraarterial application of BH_4. BH_4 doses infused in 0.9% NaCl were 11mg/kg in experiment 1, 20 mg/kg in experiment 2.

measured urinary cGMP. Our findings evidenced that the intraarterial BH_4 has an effect on the NOS - guanylate cyclase system in vivo because urinary cGMP excretion increased 3-4 times in response to BH_4 (Fig.4). The assumption that NOS is involved was further confirmed by the finding that L-arginine was consumed locally during infusion of BH_4 into the brachial artery (Fig. 5A, steps I-IV). Systemic L-arginine levels, and the L-histidine concentration in the blood effluent from the perfused limb remained unchanged (not shown). Local consumption of L-arginine by NOS in the perfused limb correlated with enhanced cGMP concentrations in the perfused limb during BH_4 infusion (Fig. 5B, steps I-IV).

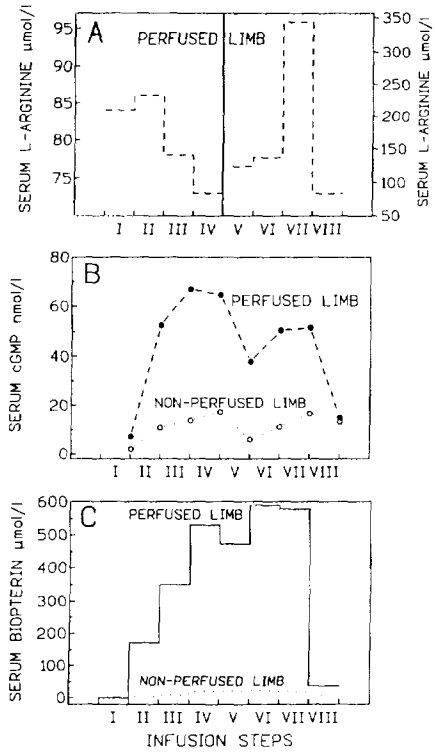


FIGURE 5.

Metabolic resonses to infusion of graded concentrations of BH_4, (steps I-IV), BH_4 plus L-NMMA (step V), or BH_4 and graded doses of L-arginine (steps VI-VII), into the Arteria brachialis, as described in Fig. 3. (A) Changes of L-arginine concentrations in the venous blood from the perfused limb. **(B)** cGMP concentrations in the venous blood from the perfused and the non-perfused limb. **(C)** BH_4 in the venous blood from the perfused and the non-perfused limb. Blood samples were drawn at the end of each infusion step through catheters inserted into both Vv. brachiales.

Infusion of graded doses of L-arginine along with BH_4 (Fig. 5A, steps VI and VII) further increased local vasodilatation (Figs. 3A and B, steps VI and VII), up to a point where edema formation started in the perfused limb, visibly by fluorescent biopterin, the extravasation product of BH_4, at the hypothenar region of the left hand (not shown). The L-arginine antagonist N^G-monomethyl-L-arginine, used at relatively low concentrations of 4 µmol/min, slightly reduced serum cGMP (Fig. 5B, step V) and skin blood flow (Fig. 3B, step V) and attenuated a further increase of vasodilatation during concomitant infusion of BH_4 (Fig. 3A, step V).

In summary, we have shown that unlike the non-polarized diffusion of NO, the highly regulated endothelial metabolite BH_4 is vectorially secreted into the direction of vascular smooth muscle cells. Smooth muscle cells, however, do not release BH_4 even when fully activated with a mixture of inflammatory cytokines and endotoxin (G. Schoedon, unpublished

observation). Infused BH_4 has marked vasodilating properties in man. In contrast to endothelial cells of animal species which express both cNOS and iNOS (21,22), human endothelial cells dispose of cNOS only (9,10,23). In the human vasculature cNOS and iNOS are compartmentalized in two different cell systems, the endothelium and the smooth muscle. The fact that BH_4 induces vasodilatation even in healthy individuals, and that in inflammatory conditions NO production by endothelial cNOS switches to smooth muscle iNOS (11) while at the same time the endothelial BH_4 output is highly enhanced let us propose that BH_4 could account for another, inflammatory EDRF.

Furthermore BH_4 could become an important target for pharmacological interventions aimed at controlling excessive vasodilatation by inhibiting its biosynthesis during septic shock, or using its vasodilating principle in pathological conditions caused by vasoconstriction e.g. the adult respiratory distress syndrome (24).

ACKNOWLEDGMENTS

We thank L. Kierat, M.Schiesser, U. Redweik for excellent technical assistance, Dr. E. Schneider for expert insertion of catheters, Dr. U. Hoffmann for expert advice, and Dr. F. Salomon for critical comments.

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