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RESEARCH COMMUNICATION

Tetrahydrobiopterin-dependent formation of endothelium-derived relaxing factor (nitric oxide) in aortic endothelial cells

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Inhibition of tetrahydrobiopterin (H_4biopterin) biosynthesis in endothelial cells almost completely abolished the agonist-induced formation of endothelium-derived relaxing factor (EDRF) (NO). This inhibitory effect could be antagonized when H_4biopterin biosynthesis was restored by activating a salvage pathway. These data indicate that the formation of EDRF strictly depends on the presence of intracellular H_4biopterin, which, in addition to Ca^{2+}, may represent a further physiological and/or pathophysiological regulatory of endothelial NO synthases.

INTRODUCTION

Endothelial cells stimulated with hormones or neurotransmitters release L-arginine-derived nitric oxide (NO) or a labile NO-containing compound (endothelium-derived relaxing factor, EDRF), which causes smooth-muscle relaxation via activation of soluble guanylate cyclase [1,2]. In mammalian tissues there are at least three isoforms of NO-generating enzymes present, all of which catalyse the conversion of L-arginine into L-citrulline and NO, but differ in the mechanisms of their regulation. Endothelial cells contain cytosolic- and membrane-associated forms of NO synthases which are both regulated by intracellular levels of free Ca^{2+} and are dependent on calmodulin [3–5]. NO synthases similar to the cytosolic endothelial enzyme are apparently present in brain [6], lung [7], adrenal gland [8] and platelets [9], and were isolated from rat and porcine cerebellum [10,11]. In addition to these constitutively expressed, Ca^{2+}-regulated enzymes ('brain-type'), there is a Ca^{2+}-independent NO synthase present in activated murine macrophages [12], and tetrahydrobiopterin (H_4biopterin) was identified as a cofactor of this enzyme [13,14]. The cofactor role of H_4biopterin in Ca^{2+}-independent NO synthesis was also demonstrated in intact cells using cultured murine fibroblasts, where a marked decrease in the cytokine-induced release of NO_2^-/NO_3^- was observed after inhibition of H_4biopterin biosynthesis [15].

The H_4biopterin-dependency of the 'brain-type' enzyme, however, is still a matter of debate. In some studies it was found that H_4biopterin shows no stimulatory effect on Ca^{2+}/calmodulin-dependent NO synthase activities [10,16,17], and the authors concluded, therefore, that NADPH represents the sole cofactor of the Ca^{2+}-regulated enzyme. In contrast with these reports, others observed an enhanced NO formation by the endothelial enzymes when H_4biopterin was present [5,18], and we found a pronounced stimulation of the isolated brain enzyme by H_4biopterin [11]. These discrepancies may be explained by our recent finding that some amounts of H_4biopterin remain bound to NO synthase in the course of protein purification [19]. Thus a lack of stimulatory effects of exogenously added H_4biopterin does not necessarily reflect a lack of H_4biopterin requirement of NO synthase, since the amount of the enzyme-bound cofactor may depend on the applied homogenization and/or purification procedures.

In the present study we investigated whether the synthesis of EDRF by hormonally stimulated porcine aortic endothelial cells depends on the presence of intracellular H_4biopterin. For these experiments, endothelial cells were preincubated with 2,4-diamino-6-hydroxypyrimidine (DAHP), a compound which reduces intracellular H_4biopterin levels by inhibiting GTP cyclohydrolase I, the rate-limiting enzyme in H_4biopterin biosynthesis [20] and/or sepiapterin, a pteridine which permits H_4biopterin biosynthesis via a salvage pathway [21]. Our data demonstrate that the formation of EDRF depends strictly on the presence of intracellular H_4biopterin and suggest that H_4biopterin represents a cofactor of endothelial NO synthase(s). Accordingly, EDRF formation and, therefore, vascular-smooth-muscle guanyl cyclase activities, may be affected or modulated by physiological and/or pathophysiological processes associated with the endothelial biosynthesis or the metabolism of reduced biopterins.

MATERIALS AND METHODS

Materials

All tissue-culture media and ingredients were from Flow Laboratories, Meckenheim, Germany, or from GIBCO/BRL G.m.b.H., Eggenstein, Germany. Tissue-culture plates and Petri dishes were obtained from Greiner, Kremsmünster, Austria. DAHP was purchased from Sigma Chemical Co., Munich, Germany, and sepiapterin was from Dr. B. Schircks Laboratories, Jona, Switzerland.

Cell culture

Porcine aortic endothelial cells were isolated by enzymic treatment (0.1% collagenase) and cultured up to two passages in Opti-MEM (GIBCO/BRL) containing 3% (v/v) foetal-calf serum and antibiotics as previously described [22]. The identity of the endothelial cells was verified by immunofluorescence (Factor VIII antigen). Before the experiments, endothelial cells were subcultured in six-well plastic plates. After reaching confluence ($\sim 10^6$ cells/dish) the culture medium was removed and Dulbecco's modified Eagle medium (Flow Laboratories) without

Abbreviations used: H_4biopterin, tetrahydrobiopterin; DAHP, 2,4-diamino-6-hydroxypyrimidine; EDRF, endothelium-derived relaxing factor; PBS, phosphate-buffered saline (0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.4); IBMX, isobutylmethylxanthine.

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Table 1. Effect of DAHP and sepiapterin (SP) of intracellular H_4biopterin levels and NO biosynthesis

Confluent monolayers of endothelial cells were preincubated for 72 h in the absence or presence of 20 mM-DAHP or/and 0.1 mM-sepiapterin (SP). Cells were washed with PBS and intracellular H_4biopterin was determined as described in the Materials and methods section. Release of NO was measured spectrophotometrically over a period of 10 min under control conditions and after stimulation with 1 μM-A23187. Data are means ± S.E.M. (n = 6–12). *$P < 0.01$ versus the respective value obtained with cells without pretreatment.

Pretreatment	H_4biopterin level (pmol/10^6 cells)	NO biosynthesis (pmol/min per 10^6 cells)	
		Control	A23187
None	0.40 ± 0.01	20.7 ± 1.58	53.8 ± 3.57
DAHP (20 mM)	< 0.05	21.0 ± 1.44	27.1 ± 2.78*
SP (0.1 mM)	248.2 ± 10.88*	19.8 ± 0.79	54.0 ± 2.30
DAHP + SP	127.6 ± 6.00*	21.5 ± 2.20	52.2 ± 1.71

serum was added, and pretreatment of endothelial cells was performed in the absence or presence of DAHP and/or sepiapterin at concentrations as indicated for 72 h.

Measurement of endothelial cyclic GMP levels

Endothelial cells were washed twice with iso-osmotic Hepes buffer, pH 7.1, containing 2.5 mM-$CaCl_2$ and 1 mM-$MgCl_2$ and preincubated for 15 min at 37 °C in 1.4 ml of the same buffer containing 1 mM-isobutylmethylxanthine (IBMX) and 1 μM-indomethacin as previously described [22]. The incubation was started by adding 100 μl of the compound to be tested and was stopped after 4 min by removal of the incubation medium and addition of 1 ml of 0.01 M-HCl. Within 1 h, intracellular cyclic GMP was completely released into the supernatant and was measured by radioimmunoassay.

Measurement of NO release

NO was determined by a spectrophotometric assay based on the NO-induced conversion of oxyhaemoglobin into methaemoglobin [23]. For these experiments, endothelial cells were washed twice with iso-osmotic Hepes buffer, pH 7.4, containing 2.5 mM-$CaCl_2$ and 1 mM-$MgCl_2$, and were equilibrated for 15 min at 37 °C. The supernatant was removed, and 1.8 ml of a solution of 5 μM-oxyhaemoglobin in the same buffer and 200 μl of the compound to be tested were added. The six-well plate was shaken for 5 s, and 1 ml of the supernatant was collected to serve as control (= oxyhaemoglobin). The incubation was then performed for 10 min at 37 °C, the supernatant removed and immediately measured against the control spectrum in a diode-array spectrophotometer (Hewlett–Packard, Vienna, Austria). The difference between the absorbances at 402 and 420 nm was recorded and NO concentrations were calculated by using a molar absorption coefficient of 56 $mM^{-1} \cdot cm^{-1}$ as determined under the conditions used in this study.

Determination of intracellular biopterin

Endothelial cells from three Petri dishes (diameter 90 mm; approx. 2×10^7 cells) were harvested, washed with iso-osmotic buffer, suspended in 700 μl of distilled water and frozen in liquid N_2. After 1 h, samples were thawed, centrifuged for 2 min at 14000 g and the supernatant split into two 300 μl portions. As previously described [15], each fraction of the sample was oxidized with 15 μl of a solution of 0.1 M-KI and 0.1 M-I_2 in the presence of 15 μl of 0.1 M-HCl or 15 μl of 0.1 M-NaOH respectively. Samples were incubated at room temperature in the dark for 1 h, mixed with 30 μl of a freshly prepared solution of ascorbic acid (0.1 M), and centrifuged for 2 min at 14000 g. The supernatant was removed and stored at −20 °C. Before analysis, pteridines were extracted with solid-phase cartridges (SCX; Varian, Palo Alto, CA, U.S.A.) and directly eluted on to a reversed-phase h.p.l.c. column (Lichrosorb RP-18; Merck, Darmstadt, Germany) by means of an automated device (AASP; Varian) as previously described [24]. Biopterin was detected by fluorescence (excitation 353 nm, emission 438 nm), and the amount of tetrahydrobiopterin calculated from the difference between biopterin after oxidation in acidic and alkaline medium respectively [25].

Statistical analysis

All experiments were performed in triplicate with at least two different batches of endothelial cells. Unless otherwise indicated, the results are expressed as means ± S.E.M. The statistical significances were evaluated by one- or two-way analysis of variance using Scheffe's F-test.

RESULTS

Stimulation of endothelial cells with 1 μM Ca^{2+}-ionophore A23187 increased the release of NO from 20.7 to 53.8 pmol/min per 10^6 cells (Table 1). Inhibition of H_4biopterin biosynthesis by pretreating endothelial cells for 72 h with 20 mM-DAHP antagonized this stimulatory effect. The diminished response of endothelial cells to A23187 correlated with reduced intracellular H_4biopterin levels. Under control conditions, a H_4biopterin level of 0.40 pmol/10^6 cells was found, whereas DAHP treatment reduced this value to less than the detection limit of 0.05 pmol/10^6 cells. To investigate a possible relationship between intracellular H_4biopterin levels and A23187-induced release of NO, we preincubated endothelial cells with DAHP and sepiapterin. As shown in Table 1, addition of 0.1 mM-sepiapterin and 20 mM-DAHP to the preincubation medium increased the intracellular concentration of H_4biopterin to 127.6 pmol/10^6 cells and completely restored the response of endothelial cells to A23187. When endothelial cells were preincubated with sepiapterin in the absence of DAHP, H_4biopterin levels were raised to 248.2 pmol/10^6 cells, but no effect of sepiapterin on the release of NO was observed.

Similar results were obtained when the formation of NO was measured as increases in intracellular cyclic GMP levels (Table 2). Under control conditions, maximally active concentrations of bradykinin and A23187 increased cyclic GMP 3.6- and 7.5-fold respectively. Sodium nitroprusside, a direct activator of soluble guanylate cyclase, raised cyclic GMP levels about 14-fold. Preincubation of endothelial cells with DAHP did not significantly affect basal cyclic GMP levels. Addition of 1 μM-A23187 to DAHP-pretreated cells slightly increased cyclic GMP levels from 0.72 to 1.45 pmol/10^6 cells, which was less than 25% of the

Table 2. Effect of DAHP and sepiapterin (SP) on intracellular cyclic GMP levels

Confluent monolayers of endothelial cells were preincubated for 72 h in the absence or presence of 20 mM-DAHP or/and 0.1 mM-sepiapterin (SP). Cells were washed with PBS, preincubated for 15 min with 1 mM-IBMX and 1 μM-indomethacin and stimulated with bradykinin (1 μM), A23187 (1 μM) or sodium nitroprusside (1 mM) for 4 min. Intracellular cyclic GMP levels were measured by radioimmunoassay as described in the Materials and methods section. Data are means ± S.E.M. ($n = 12$). *$P < 0.01$ versus the respective value obtained with cells without pretreatment.

Pretreatment	Intracellular cyclic GMP level (pmol/10^6 cells)			
	Control	Bradykinin	A23187	Nitroprusside
None	0.96 ± 0.05	3.49 ± 0.33	7.18 ± 1.45	13.64 ± 0.73
DAHP (20 mM)	0.72 ± 0.08	0.78 ± 0.09*	1.45 ± 0.10*	13.74 ± 1.33
SP (0.1 mM)	1.23 ± 0.15	4.72 ± 0.33*	8.61 ± 0.66	13.34 ± 0.82
DAHP + SP	0.97 ± 0.07	4.28 ± 0.10	8.57 ± 0.66	12.88 ± 1.53

Table 3. Effect of increasing concentrations of DAHP on intracellular H_4biopterin and cyclic GMP levels

Confluent monolayers of endothelial cells were preincubated for 72 h in the absence or presence of various concentrations of DAHP. Cells were washed with PBS, and intracellular H_4biopterin was determined as described in the Materials and methods section. For cyclic GMP measurements, cells were preincubated for 15 min with 1 mM-IBMX and 1 μM-indomethacin and incubated for 4 min more in the absence or presence of A23187 (1 μM). Intracellular cyclic GMP levels were measured by radioimmunoassay as described in the Materials and methods section. Data are means ± S.E.M. ($n = 3$–6). *$P < 0.01$ versus the respective value obtained with cells without pretreatment.

Pretreatment (mM-DAHP)	H_4biopterin level (pmol/10^6 cells)	Cyclic GMP level (pmol/10^6 cells)	
		Control	A23187
0	0.61 ± 0.03	1.02 ± 0.04	5.00 ± 0.18
0.3	0.18 ± 0.01*	0.90 ± 0.04	4.96 ± 0.18
1	0.10 ± 0.01*	0.78 ± 0.04*	3.11 ± 0.19*
3	< 0.05	0.69 ± 0.05*	2.57 ± 0.10*
10	< 0.05	0.64 ± 0.04*	1.77 ± 0.07*
20	< 0.05	0.71 ± 0.06*	1.47 ± 0.05*

A23187 effect obtained under control conditions. Bradykinin, which produces in cultured endothelial cells a considerably lower NO formation than A23187, completely failed to stimulate cyclic GMP production in DAHP-treated cells. Direct activation of soluble guanylate cyclase by sodium nitroprusside, however, was unaffected by DAHP treatment, which clearly demonstrates that DAHP did not exert its effect via inhibition of guanylate cyclase. Addition of sepiapterin to the preincubation medium completely antagonized the inhibitory effects of DAHP on bradykinin- and A23187-stimulated cyclic GMP production.

In a further set of experiments, endothelial cells were preincubated in the presence of increasing concentrations of DAHP. As Table 3 shows, DAHP at a concentration of 0.3 mM reduced endothelial H_4biopterin levels from 0.61 to 0.18 pmol/10^6 cells, but did not significantly affect cyclic GMP production. At higher concentrations DAHP further reduced H_4biopterin levels below the detection limit and concomitantly decreased cyclic GMP levels in a concentration-dependent manner with an IC_{50} (concn. causing 50% of maximal inhibitory effect) value of about 1 mM.

DISCUSSION

H_4biopterin was found to stimulate cytokine-inducible NO synthase obtained from murine macrophages [13,14] and was recently described as modulating cytokine-induced NO biosynthesis in intact fibroblasts [15]. The possibility of a regulatory role for H_4biopterin in Ca^{2+}/calmodulin-dependent NO biosynthesis, however, is still under discussion. Here we demonstrate, using intact endothelial cells, that H_4biopterin is essential for the Ca^{2+}-induced formation of NO, which was measured as release of NO from the cells as well as increase in intracellular cyclic GMP levels [22,26].

Inhibition of H_4biopterin biosynthesis by DAHP [20] not only reduced intracellular H_4biopterin levels, but also almost completely abolished the effects of bradykinin and A23187 on NO formation. The inhibitory effect of DAHP on intracellular H_4biopterin levels was directly linked to a diminished NO production, since enhancing H_4biopterin levels by sepiapterin completely restored the formation of NO. Concentration–response studies with DAHP revealed that a reduction of H_4biopterin levels to less than 30% of the respective control was required to reduce significantly the formation of NO. In contrast with Ca^{2+}-stimulated NO formation, basal NO biosynthesis is less sensitive to DAHP inhibition, indicating that the cells can keep basal NO formation with only small amounts of H_4biopterin.

Our data demonstrate that intracellular concentrations of H_4biopterin modulate the amount of NO formed by endothelial cells. Since a continuous release of endothelial NO is apparently necessary to maintain a lowered blood pressure *in vivo* [27], our data provide an explanation of the molecular mechanism that may link the observed reduced capacity of H_4biopterin biosynthesis and the increase of blood pressure in spontaneously hypertensive rats [28]. In addition to those observed in neurological disorders, declines in H_4biopterin synthesis or recycling have also been observed to occur, e.g., with age or in metal poisoning [29–32]. Although it remains to be seen to what extent endothelial cells are responsible for and/or afflicted by these declines, our results clearly suggest that alterations in H_4biopterin levels may alter the formation of endothelium-derived NO also *in vivo*. This offers a rationale for a new approach to therapeutic intervention into EDRF biosynthesis by drugs influencing pteridine metabolism.

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