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Cell type-specific recycling of tetrahydrobiopterin by dihydrofolate reductase explains differential effects of 7,8-dihydrobiopterin on endothelial nitric oxide synthase uncoupling

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ABSTRACT

(6R)-5,6,7,8-Tetrahydro-L-biopterin (BH4) availability regulates nitric oxide and superoxide formation by endothelial nitric oxide synthase (eNOS). At low BH4 or low BH4 to 7,8-dihydrobiopterin (BH2) ratios the enzyme becomes uncoupled and generates superoxide at the expense of NO. We studied the effects of exogenously added BH2 on intracellular BH4/BH2 ratios and eNOS activity in different types of endothelial cells. Incubation of porcine aortic endothelial cells with BH2 increased BH4/BH2 ratios from 8.4 (controls) and 0.5 (BH4-depleted cells) up to ~20, demonstrating efficient reduction of BH2. Uncoupled eNOS activity observed in BH4-depleted cells was prevented by preincubation with BH2. Recycling of BH4 was much less efficient in human endothelial cells isolated from umbilical veins or derived from dermal microvessels (HMEC-1 cells), which exhibited eNOS uncoupling and low BH4/BH2 ratios under basal conditions and responded to exogenous BH2 with only moderate increases in BH4/BH2 ratios. The kinetics of dihydrofolate reductase-catalyzed BH4 recycling in endothelial cytosols showed that the apparent BH2 affinity of the enzyme was 50- to 300-fold higher in porcine than in human cell preparations. Thus, the differential regulation of eNOS uncoupling in different types of endothelial cells may be explained by striking differences in the apparent BH2 affinity of dihydrofolate reductase.

1. Introduction

The pterin derivative tetrahydrobiopterin (BH4) is essential for endothelial NO formation. It binds to endothelial nitric oxide synthase (eNOS) in immediate vicinity of the heme and promotes the transfer of electrons from the reductase domain to the heme group, where oxygen is reduced and incorporated into the guanidine group of the substrate, L-arginine. At subsaturating concentrations of BH4, autoxidation of the ferrous-oxy and ferrous-superoxy complexes results in release of superoxide anion and hydrogen peroxide at the expense of the L-citrulline and NO [1]. This phenomenon, referred to as eNOS uncoupling, has been implicated in endothelial dysfunction associated with a variety of cardiovascular diseases including atherosclerosis, hypertension and diabetes [2–4]. Over the past several years, evidence has accumulated that in addition to BH4 levels, the intracellular ratio of BH4 *vs.* its oxidation product, dihydrobiopterin (BH2), also determines endothelial function. BH2 competes with BH4 for binding to eNOS but does no provide electrons for reductive oxygen activation [5–7]. Accordingly, eNOS uncouples at low BH4/BH2 ratios even at sufficiently high BH4 concentrations.

Intracellular levels of BH4 and BH4/BH2 ratios are controlled by *de novo* and salvage pathways [8,9]. Guanosine triphosphate cyclohydrolase I (GTPCH) is the first and rate-limiting enzyme in the *de novo* biosynthetic pathway. It catalyzes the hydrolysis of GTP to 7,8-dihydroneopterin triphosphate, which is then converted to BH4 by the consecutive action of 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase. GTPCH activity, and thus *de novo* synthesis of BH4 is primarily regulated at the transcriptional level by several cytokines and hormones, which either up- or down-regulate GTPCH expression levels, as well as by

Abbreviations: amino-BH4, 4-amino-(6R)-5,6,7,8-tetrahydro-L-biopterin; BAECs, bovine aortic endothelial cells; BH2, 7,8-dihydro-L-biopterin; BH4, (6R)-5,6,7,8-tetrahydro-L-biopterin; cGMP, 3′,5′-cyclic guanosine monophosphate; DAHP, 2,4-diamino-6-hydroxypyrimidine; DEA/NO, 2,2-diethyl-1-nitroso-oxyhdrazine; DHF, 7,8-dihydrofolate; DHFR, dihydrofolate reductase; eNOS, endothelial nitric oxide synthase; GTPCH, guanosine triphosphate cyclohydrolase I; HUVECs, human umbilical vein endothelial cells; L-NMA, N^G-methyl-L-arginine; L-NNA, N^G-nitro-L-arginine; NO, nitric oxide; PAECs, porcine aortic endothelial cells; ROS, reactive oxygen species; THF, 5,6,7,8-tetrahydrofolate.

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posttranslational modifications and by interaction of GTPCH with its feedback regulatory protein. Moreover, GTPCH activity can be efficiently inhibited by 2,4-diamino-6-hydroxypyrimidine (DAHP), a pharmacological tool used for the depletion of cellular BH4.

In addition to its *de novo* biosynthesis, BH4 can be enzymatically regenerated from its oxidation products quinonoid 6,7-[8H]-dihydrobiopterin and 7,8-dihydrobiopterin (BH2) by dihydropteridine reductase and dihydrofolate reductase (DHFR), respectively. While the role of dihydropteridine reductase in maintaining endothelial function is unclear (the quinonoid 6,7-[8H]-dihydrobiopterin rearranges non-enzymatically to BH2, which is then reduced to BH4 by DHFR), inhibition or knockout of DHFR in cultured endothelial cells has been shown to reduce intracellular BH4:BH2 ratios and NO/L-citrulline formation [10–12], hinting at a critical role of DHFR in regulating eNOS uncoupling. More recently the results obtained with cultured cells have been corroborated by *in vivo* experiments showing that treatment of BH4-deficient mice with the DHFR inhibitor methotrexate induces reduction of BH4:BH2 ratios und eNOS uncoupling in lung tissue [13].

As demonstrated with human aortic endothelial cells, bovine aortic endothelial cells (BAECs) and the murine endothelial cell line sEnd.1, the capacity of DHFR in reducing BH2 to BH4 is apparently rather low, as the cells respond to extracellular BH2 with a substantial increase in intracellular BH2, diminished NO and enhanced superoxide formation even if DHFR is not inhibited or knocked out [5,12,14]. These findings showing that supplementation of cells with BH2 induces eNOS uncoupling were in striking contrast to our preliminary observation that BH2 restores eNOS function in BH4-depleted porcine aortic endothelial cells (PAECs).

The present study was aimed at clarifying whether cell type-specific differences in BH2-to-BH4 reduction may account for the differential effects of BH2 supplementation on eNOS function.

2. Materials and methods

2.1. Materials

L-[2,3-^{3}H]Arginine hydrochloride (1.5–2.2 TBq/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA) and purified as described earlier [15]. DEA/NO was obtained from Alexis Corporation (Lausen, Switzerland) and dissolved and diluted in 10 mM NaOH. Dihydroethidium was from Calbiochem – Merck4Biosciences (Darmstadt, Germany) and dissolved in DMSO. BH4, BH2 and amino-BH4 were from Schircks Laboratories (Jona, Switzerland). Antibiotics and fetal calf serum were purchased from PAA Laboratories (Linz, Austria). Culture media and other chemicals were from Sigma–Aldrich (Vienna, Austria).

2.2. Culture and treatment of endothelial cells

Porcine aortic endothelial cells (PAECs) were isolated as described [16] and cultured at 37 °C, 5% CO_2, in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 μg/ml amphotericin B. Human umbilical vein endothelial cells (HUVECs) were isolated as described [17] and cultured in Medium 199, supplemented with 15% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1.25 μg/ml amphotericin B, 2 mM L-glutamine, 5000 U/ml heparin, and 10 μg/ml endothelial cell growth factor. The human microvascular endothelial cell line, HMEC-1 [18] was kindly provided by F.J. Candal (Centers for Disease Control, Atlanta, GA, USA) and was maintained in medium MCDB131 supplemented with 15% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1.25 μg/ml amphotericin B, 10 ng/ml epidermal growth factor, and 1 mg/ml hydrocortisone. Where indicated, cells were pretreated in culture medium containing DAHP, aminopterin and/or pteridines.

2.3. Determination of endothelial L-[^{3}H]citrulline formation

Intracellular conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline was measured as previously described [19]. Briefly, cells grown in 6-well plates were washed and equilibrated for 15 min at 37 °C in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 2.5 mM $CaCl_2$ (incubation buffer). Reactions were started by addition of L-[2,3-^{3}H]arginine (~10^6 dpm) and A23187 (1 μM) and terminated after 10 min by washing the cells with chilled incubation buffer. Subsequent to lysis of the cells with 0.01 N HCl, an aliquot was removed for the determination of incorporated radioactivity. To the remaining sample, 200 mM sodium acetate buffer (pH 13.0) containing 10 mM L-citrulline was added (final pH ~5.0), and L-[^{3}H]citrulline separated from L-[^{3}H]arginine by cation exchange chromatography [19].

2.4. Determination of endothelial cGMP formation

Accumulation of intracellular cGMP was determined as previously described [19]. Briefly, cells grown in 24-well plates were washed and preincubated for 15 min at 37 °C in incubation buffer (see above), additionally containing 1 mM 3-isobutyl-1-methylxanthine and 1 μM indomethacin. Reactions were started by addition of A23187 (1 μM) and terminated after 4 min by removal of the incubation medium and addition of 0.01 N HCl. Within 1 h, intracellular cGMP was completely released into the supernatant and measured by radioimmunoassay.

2.5. Determination of endothelial reactive oxygen species (ROS) formation

Cells grown on glass coverslips were incubated for 10 min at 37 °C in incubation buffer containing 10 μM dihydroethidium, washed and incubated for further 30 min with buffer containing 1 μM A23187. Alternatively, cells were incubated for 4 h in phenol red free culture medium containing 10 μM dihydroethidium and, where indicated, stimulated with 1 μM A23187 1 h prior to the end of incubation. The latter method allowed a more reliable measurement of basal ROS formation and was used for the experiments shown in Fig. 5. Subsequent to incubation, cells were washed with incubation buffer and fluorescence imaging was performed at room temperature using a Zeiss Axiovert 200 setup, equipped with a xenon lamp, polychromator, Chroma filters (exciter: HQ546/12; emitter: HQ605/75; beamsplitter: Q560lp) and a CoolSNAP fx-HQ CCD-camera (Visitron Systems GmbH, Puchheim, Germany). The microscopic fields were selected randomly and the exposure time was 5 s for each image. Routinely, 3 images were taken and at least 50 cells/coverslip were analyzed using the Meta Imaging Series 5.0 software from Universal Imaging Corporation (Downingtown, PA, USA) or the open source image analysis program, ImageJ (http://imagej.nih.gov/ij/). Values were corrected for background fluorescence measured in cell-free areas.

2.6. Determination of BH4, BH2 and biopterin levels

Intracellular levels of pteridines were quantified by HPLC analysis using a method adapted from Fukushima and Nixon [20] as described previously [21]. Briefly, cells grown in petri dishes (diameter 90 mm) were harvested (~5×10^6 per petri dish), washed, and resuspended in either 0.1 ml of PBS containing 1 mM DTT (for quantifying biopterin), 0.1 ml of alkaline oxidant solution (0.02 M KI/I_2 in 0.1 M NaOH; for quantifying biopterin plus BH2), or 0.1 ml of acidic oxidant solution (0.02 M KI/I_2 in 0.1 M HCl; for

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