Regulation of 6-Pyruvoyltetrahydropterin Synthase Activity and Messenger RNA Abundance in Human Vascular Endothelial Cells

Philippe Linscheid, MSc; Andreas Schaffner, MD; Nenad Blau, PhD; Gabriele Schoedon, PhD

Background—The nitric oxide synthase cofactor tetrahydrobiopterin (BH4) is involved in the regulation of endothelium-dependent vascular functions mediated by nitric oxide. Vascular endothelial cells synthesize and secrete large amounts of BH4 on cytokine activation. There is scant knowledge about molecular mechanisms of cytokine-triggered BH4 production in endothelial cells.

Methods and Results—Pteridine production, mRNA expression of GTP cyclohydrolase (GTPCH) and 6-pyruvoyltetrahydropterin synthase (PTPS) (both key enzymes of BH4 biosynthesis), and PTPS activity were studied in human umbilical vein endothelial cells (HUVECs) exposed to inflammatory cytokines. BH4 levels were 140-fold enhanced on treatment of HUVECs with combined interferon-γ/tumor necrosis factor-α/interleukin-1 (IFN/TNF/IL-1). Specific PTPS activity was 3-fold higher in cytokine-treated HUVECs than in untreated cells. Reverse-transcription limiting-dilution polymerase chain reaction analysis showed that in response to IFN/TNF/IL-1, mRNA abundance of GTPCH and PTPS was increased 64-fold and 10-fold, respectively.

Conclusions—The present study demonstrates for the first time the cytokine-dependent regulation of PTPS, the second enzyme in BH4 synthesis. Although GTPCH is believed to be the rate-limiting step, control of endothelial PTPS expression by cytokines may play an important role in regulating BH4-dependent nitric oxide production in the vascular system. (Circulation. 1998;98:1703-1706.)

Key Words: cells ■ endothelium-derived factors ■ enzymes ■ vasculature

Nitric oxide (NO) has emerged as an important factor controlling vascular tone under normal and pathological conditions. There is increasing evidence that tetrahydrobiopterin (BH4), the natural cofactor of NO synthases, plays a crucial role in the regulation of NO synthesis in vascular cells. Intracellular BH4 availability modulates NO-mediated cGMP production in human endothelial cells, whereas BH4 synthesis is regulated by inflammatory and anti-inflammatory cytokines. Interestingly, inflammatory cytokines enhance BH4 synthesis and constitutive NO synthase (cNOS) specific activity, with a concomitant decrease of cNOS enzyme in human vascular endothelial cells. In vascular smooth muscle cells (SMCs), inducible NO synthase (iNOS) is upregulated by inflammatory cytokines, and therefore BH4 becomes limiting for maximal iNOS activity. GTP cyclohydrolase I (GTPCH), the first enzyme in the biosynthesis of BH4, is regulated by cytokines and is commonly thought to be the rate-limiting enzyme. However, 6-pyruvoyltetrahydropterin synthase (PTPS), the second enzyme in the biosynthesis of BH4, can become rate limiting on induction of GTPCH, especially in human cells, as reflected by neopterin accumulation. Human endothelial cells express GTPCH activity and synthesize small amounts of BH4 constitutively. Under inflammatory conditions, endothelial cells synthesize and secrete large amounts of BH4 and little or no neopterin, indicating that PTPS is not rate limiting in these cells. Recently, GTPCH mRNA induction by cytokines has been reported in human endothelial cells, but nothing is known about regulation of PTPS in those cells. Because vascular endothelial cells are the main producers of BH4 in physiological and pathological conditions, we investigated the expression of GTPCH and PTPS, the key enzymes of BH4 biosynthesis, at the molecular level in human umbilical vein endothelial cells (HUVECs) under normal and inflammatory conditions.

Methods

Cell Culture

HUVECs and human aortic SMCs (ATCC) were maintained in humidified air, 5% CO₂ at 37°C. Cells from passages 2 to 4 were seeded on 100-mm culture dishes in 10 mL of medium. At confluence, cells were incubated with interferon-γ (IFN-γ; 100 U/mL), tumor necrosis factor-α (TNF-α; 100 U/mL), and interleukin-1 (IL-1; 20 U/mL) as indicated.
Regulation of PTPS Activity and Pteridine Synthesis in HUVECs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PTPS Activity, µU/mg Protein</th>
<th>Neopterin, pmol/mg Cell Protein</th>
<th>Biopterin, pmol/mg Cell Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.59±0.05*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ (100 U/mL)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>TNF-α (100 U/mL)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>IL-1 (20 U/mL)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Cyt-mix</td>
<td>1.74±0.03*</td>
<td>3.18‡</td>
<td>25‡</td>
</tr>
</tbody>
</table>

ND indicates not detectable. Pterins and PTPS activity were measured as described in “Methods.”

‡P<0.01 by 2-tailed t test. Mean±SD from 1 representative experiment.

†P<0.02 by ANOVA with Dunnett’s correction for multiple comparisons with 1 control. Mean±SEM from triplicate independent experiments.

Single determination.

Reverse Transcriptase–Polymerase Chain Reaction
The relative expression of GTPCH, PTPS, cNOS, and iNOS mRNAs compared with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was evaluated in control and cytokine-treated cells. Aliquots containing 5 µg of total cellular RNA were reverse transcribed, and first-strand cDNA was used as a template in polymerase chain reaction (PCR). cDNA aliquots were amplified with the following specific primers: PTPS, 5'-TGCTGTCACAGTTTGCT-3' (sense) and 5'-CCACAGTATATTATGTGCTG-3' (antisense), yielding a 397-bp product located at base pairs 282 to 931 of the iNOS sequence (Genebank M97655); GTPCH, 5'-TGGTTATCCTTCAACAA-3' (sense) and 5'-GTGCTGTCACAGTTTGCT-3' (antisense), yielding a 226-bp product located at base pairs 460 to 686 of the GTPCH sequence (Genebank U19523); cNOS, 5'-CACGGCCTACTACCTGAGG-3' (sense) and 5'-CTGGTTACTGCGCTCTCC-3' (antisense), yielding a 799-bp product located at base pairs 1130 to 1929 of the cNOS sequence (Genebank M95296); and iNOS, 5'-GGACATCAACAACGTAACAA-3' (sense) and 5'-CTTGTTACTGCGCTCTCC-3' (antisense), yielding a 649-bp product located at base pairs 282 to 931 of the iNOS sequence (Genebank L09210). Primers specific for human GAPDH were from Stratagene. The origin of amplified PCR products from cDNAs of GTPCH, PTPS, cNOS, and iNOS, respectively, was confirmed by sequencing. PCR products in agarose gels (Visigel matrix, Stratagene) were analyzed by use of the Bio-Rad Gel Doc 1000 System.

Comparative Measurement of mRNA Levels
For comparative determination of mRNA levels, limiting-dilution PCRs of cDNA were performed.15,16 After reverse transcription (RT) as described above, equal amounts of cDNA were serially diluted 2-fold up to 256-fold and cycled as described above.

 Determination of PTPS Activity
Specific PTPS activity was measured after 48 hours as described previously.15

Measurement of Pterins
Pterins were determined after 24 hours by high-performance liquid chromatography after acidic oxidation as described previously.16

Statistical Analysis
Statistical analysis was performed by ANOVA with Dunnett’s correction for comparison of multiple groups with 1 control by use of a computer-based program (Instat 3.0, Graphpad).

Results
The effects of inflammatory cytokines on pteridine synthesis and specific PTPS activity in HUVECs are shown in the Table. Inflammatory cytokines predominantly caused enhancement of BH4 and no or only moderate increase of neopterin. A 140-fold increase of extracellular BH4 (20 to 2920 pmol/mg) was found on incubation with mixed cytokines. Under these conditions, extracellular BH4 (2920 pmol/mg) was ≥116-fold higher than the corresponding intracellular portion (25 pmol/mg), indicating that the major part of the cofactor is secreted.

PTPS activity was constitutively present in untreated HUVECs. After incubation of HUVECs with IFN-γ/TNF-α/IL-1, specific PTPS activity was enhanced 3-fold (Table).

To investigate to what extent these metabolic parameters reflect regulation at the molecular level, we examined the effects of inflammatory cytokines on mRNA expression of GTPCH and PTPS, the key enzymes of BH4 de novo synthesis. In parallel, cNOS and iNOS mRNAs were also investigated. The results of RT-PCR are shown in panel A of the Figure. In untreated HUVECs, mRNAs of all enzymes except iNOS were constitutively present, GTPCH and PTPS in low amounts and cNOS in higher amounts. Treatment with IFN-γ and TNF-α enhanced GTPCH mRNA abundance, whereas IFN-γ and TNF-α alone had no effect on PTPS mRNA. In contrast, IL-1 alone did not influence GTPCH mRNA but significantly enhanced PTPS mRNA. Combination of the 3 inflammatory cytokines further enhanced GTPCH mRNA and, most interestingly, PTPS mRNA abundance. Under inflammatory conditions, cNOS mRNA was diminished and GAPDH mRNA levels remained unchanged. iNOS mRNA was not detectable in HUVECs. In human vascular SMCs, however, large amounts of iNOS mRNA were expressed after treatment with IFN-γ/TNF-α/IL-1 (Figure).

The extent of regulation of GTPCH, PTPS, and cNOS mRNAs in HUVECs was evaluated by comparative limiting-dilution PCR analysis of the respective cDNAs (panel B of the Figure). In cells treated with mixed inflammatory cytokines, PTPS mRNA abundance was ≥10-fold higher than in untreated cells. mRNA of GTPCH was ≥64-fold enhanced, whereas mRNA of cNOS declined to undetectable levels.

Discussion
In the present report, the regulation of mRNA abundance and specific activity of PTPS, the second enzyme in the synthesis of BH4, by inflammatory cytokines is described for the first time. GTPCH, the first enzyme in the BH4 synthesis path-
way, has been widely described as the rate-limiting step in mammals. Up to a 40-fold regulation of GTPCH activity was found in cells treated with inflammatory stimuli, which is consistent with the strongly elevated GTPCH mRNA level reported in the present article. In humans, PTPS was generally believed to be constitutively present and to become rate limiting on induction of GTPCH, reflected by production of higher neopterin than biopterin concentrations. However, the persistent low levels of neopterin in HUVECs even after inflammatory activation (Table), suggest that PTPS is either present with high constitutive activity or that its expression is upregulated by cytokines. We demonstrated that PTPS is upregulated in HUVECs by a combination of inflammatory cytokines not only by measuring PTPS enzyme activity using an assay system generally approved for diagnosis of PTPS deficiencies, but also by RT-PCR analysis of PTPS mRNA abundance. IL-1 seems to be the leading signal for PTPS regulation in HUVECs, because it is effective as a single stimulus and strongly synergizes with IFN-γ/TNF-α (Figure), each of which was ineffective alone. This finding is in accordance with a previous report on PTPS activity in HUVECs in which IFN-γ and TNF-α did not alter PTPS activity. Although cytokine regulation of PTPS is a novel finding in cells of the vascular system, regulation of PTPS mRNA abundance has been described in other organs: a 3- to 4-fold elevation of PTPS mRNA has been reported in rat adrenal gland after treatment with reserpine. Furthermore, both GTPCH and PTPS enzyme activities have been described to be coordinately induced by cytokines in T cells.

The mechanism behind the upregulation of PTPS mRNA by cytokines in HUVECs remains to be elucidated. However, our observation demonstrates on the molecular level the relationship between endothelial BH4 production and iNOS expression in SMCs (Figure), substantiating previous findings concerning human vascular cell functions: cNOS and iNOS are compartmentalized in endothelium and SMCs, and there is a coordinated switching of NO production from endothelial cNOS to smooth muscle iNOS in the presence of inflammatory cytokines. Because BH4 is limiting for maximal NO production in SMCs, BH4 secreted by activated endothelial cells could serve as an additional cofactor necessary for prolonged NO production for iNOS expressed in adjacent SMCs.

References

Regulation of 6-Pyruvoyltetrahydropterin Synthase Activity and Messenger RNA Abundance in Human Vascular Endothelial Cells
Philippe Linscheid, Andreas Schaffner, Nenad Blau and Gabriele Schoedon

Circulation. 1998;98:1703-1706
doi: 10.1161/01.CIR.98.17.1703

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/98/17/1703

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/