

### Critical Role of Interleukin-1 $\beta$ for Transcriptional Regulation of Endothelial 6-Pyruvoyltetrahydropterin Synthase

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**Objective**—Synthesis of tetrahydrobiopterin (BH<sub>4</sub>), an essential cofactor for nitric oxide synthases, is strongly induced on immunostimulation in vascular endothelial cells (VECs). Expression of GTP cyclohydrolase I (GTPCH), the first enzyme in BH<sub>4</sub> biosynthesis, is regulated by cytokines and considered rate-limiting. Herein we investigated the molecular mechanism and relevance of cytokine-dependent regulation of 6-pyruvoyltetrahydropterin synthase (PTPS), the second enzyme in BH<sub>4</sub> synthesis, in human coronary artery endothelial cells (HCAECs).

**Methods and Results**—Real-time polymerase chain reaction revealed a 4-fold induction of PTPS and a 300-fold induction of GTPCH expression by interleukin (IL)-1 $\beta$ /tumor necrosis factor- $\alpha$ /interferon- $\gamma$ , mainly through de novo transcription. On immunostimulation, PTPS became rate-limiting. Importantly, IL-1 $\beta$  induced PTPS rather than GTPCH. As a result, IL-1 $\beta$  contributed significantly to the amount of BH<sub>4</sub> produced (+40%) but concomitantly reduced the accumulation of the GTPCH intermediate, 7,8-dihydroneopterin triphosphate (–50%).

**Conclusion**—Our data show that PTPS induction is necessary for optimized BH<sub>4</sub> synthesis in cytokine-stimulated HCAECs and point to IL-1 $\beta$  as a leading cytokine in this process. (*Arterioscler Thromb Vasc Biol.* 2003;23:e50-e53.)

**Key Words:** endothelium ■ interleukin-1 $\beta$  ■ nitric oxide synthase ■ tetrahydrobiopterin

Recent studies have established a pivotal regulatory role for tetrahydrobiopterin (BH<sub>4</sub>), an essential cofactor for nitric oxide synthases (NOSs).<sup>1,2</sup> In the vascular system, BH<sub>4</sub> is mainly produced by endothelial cells (VECs), which secrete small amounts of BH<sub>4</sub> under noninflammatory conditions, whereas strongly enhanced BH<sub>4</sub> secretion is triggered on immunostimulation and might serve underlying smooth muscle cells for maximal NO production.<sup>3</sup>

De novo BH<sub>4</sub> biosynthesis sequentially involves GTP cyclohydrolase I (GTPCH), 6-pyruvoyltetrahydropterin synthase (PTPS), and sepiapterin reductase (SR).<sup>4</sup> GTPCH activity is considered rate-limiting and is highly induced by inflammatory cytokines. By comparison, PTPS expression is constitutive and regulated to only a very limited degree.<sup>4</sup> Nevertheless, it has long been appreciated that PTPS might become rate-limiting in some cell types after immunostimulation of GTPCH; eg, stimulated human monocytes/macrophages form little BH<sub>4</sub> but instead release high amounts of neopterin, a side product derived from the GTPCH product 7,8-dihydroneopterin triphosphate, because of very low PTPS activity.<sup>5</sup>

VECs secrete low but detectable amounts of neopterin on immunostimulation, indicating that PTPS activity is almost sufficient for BH<sub>4</sub> synthesis in these cells.<sup>3</sup> Although mRNA abundance and activity of PTPS are a fewfold enhanced on cytokine stimulation in human umbilical vein endothelial cells (HUVECs),<sup>6</sup> the importance of this regulation remained unclear. We now show that enhanced PTPS expression is necessary for optimized BH<sub>4</sub> synthesis in cytokine-stimulated VECs and provide evidence that interleukin (IL)-1 $\beta$  plays a key role for PTPS induction.

#### Methods

##### Cell Culture

Primary human coronary artery ECs from single donors (HCAECs; Clonetics) were cultured in a microvascular endothelial cell medium (EGM-2-MVBulletkit, Clonetics; contains ascorbic acid as well as human endothelial growth factor, human fibroblast growth factor-B, vascular endothelial growth factor, and R<sup>3</sup>-insulinlike growth factor-1). Cells from passages 3 to 6 were seeded in T-75 flasks or 6-well plates (BD Biosciences). At 90% confluence, cells were incubated with fresh medium containing human recombinant (hr) IL-1 $\beta$  (20 U/mL), hr-tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , 100 U/mL), and hr-

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interferon- $\gamma$  (IFN- $\gamma$ , 100 U/mL; all from Pepro Tech) in various combinations for up to 90 hours.

### Quantitative Real-Time PCR

Total RNA was isolated with use of a commercially available kit (QIAGEN RNeasy Mini kit) with DNase digestion. Total RNA (1  $\mu$ g) was reverse-transcribed into cDNA (ProSTAR first-strand kit, Stratagene) and amplified by real-time polymerase chain reaction (PCR; Light Cycler, Roche Diagnostics) with the Fast Start DNA Master SYBR Green (Roche Diagnostics). Aliquots of cDNA were amplified with the following specific primers: for PTPS, 5'-CGAGCCACCGATTGTACAGTAA-3' (forward) and 5'-GTTGTCCCAGATATAAACAGCTACA-3' (reverse); for GTPCH, 5'-TGGTTATCTTCCTAACAAG-3' (forward) and 5'-GTGCTGTCCACAGTTTGTCT-3' (reverse); for SR, 5'-CTGAACCTGACCTCCATGC-3' (forward) and 5'-CTTGAACCTCGTCTTTTCC-3' (reverse); for endothelial NOS (eNOS), 5'-AATCCTGTATGGCTCCGAGA3' (forward) and 5'-ATGCTGTTGAAGCGGATCTT-3' (reverse); and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-GGAAGGTGAAGGTCCGAGTCAACGG-3' (forward) and 5'-TCCTGGAAGATGGTGATGGGATTC-3' (reverse).

Cycling conditions were as follows: initial denaturation (10 minutes at 95°C) followed by 45 cycles of denaturation (15 seconds at 95°C), annealing (10 seconds at 55°C to 67°C), and extension (12 seconds at 72°C), with acquisition of fluorescence after each extension. PCR products were sequenced to verify specific amplification. Fivefold serial dilutions of purified PCR products (Quiaquick PCR purification kit, Qiagen) were prepared as external standards, and mRNA expression was quantified in duplicate samples as described previously.<sup>7</sup> Specific gene expression was normalized for the amount of GAPDH mRNA and expressed as fold induction in treated compared with untreated cells; quantitative studies confirmed that the amount of GAPDH mRNA per microgram of total RNA did not change in cells treated with different cytokines.

### Measurement of Pterins

Pterins in culture supernatants were determined by high-performance liquid chromatography after acidic oxidation.<sup>8</sup>

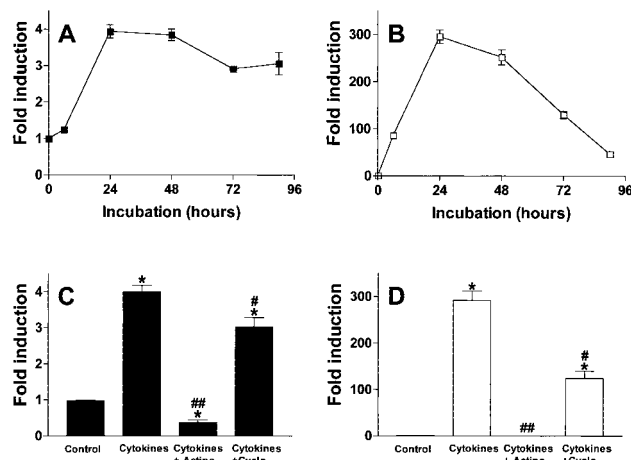
### Statistical Analysis

Results are expressed as mean  $\pm$  SEM or SD, as indicated. Data were analyzed with unpaired 2-tailed Student's *t* test with available software (Instat 3.0, GraphPad);  $P < 0.05$  was considered significant.

## Results

Incubation of HCAECs with IFN- $\gamma$ /TNF- $\alpha$ /IL-1 $\beta$  resulted in increased PTPS mRNA levels that peaked after 24 hours and then gradually decreased during the next 66 hours (Figure 1A). As expected,<sup>4</sup> cytokine treatment also strongly induced GTPCH (Figure 1B). In contrast, SR mRNA levels did not change significantly on cytokine treatment for up to 72 hours (maximal change,  $\pm 1.3$ -fold). Accumulation of biopterin, the fully oxidized form of BH<sub>4</sub>, and neopterin, an oxidized form of 7,8-dihydroneopterin triphosphate, was measured in supernatants to study the effect of cytokines on pteridine synthesis. Whereas only little biopterin and no neopterin were produced by unstimulated HCAECs, large amounts of biopterin and detectable amounts of neopterin were secreted by IFN- $\gamma$ /TNF- $\alpha$ /IL-1 $\beta$ -stimulated cells (see following paragraphs).

As shown in Figure 1C, simultaneous incubation with the transcription inhibitor actinomycin D (1  $\mu$ g/mL) for 24 hours not only prevented an IFN- $\gamma$ /TNF- $\alpha$ /IL-1 $\beta$ -induced increase in PTPS mRNA abundance but also resulted in significantly lower PTPS mRNA levels compared with untreated cells and completely prevented the increase in GTPCH mRNA levels.

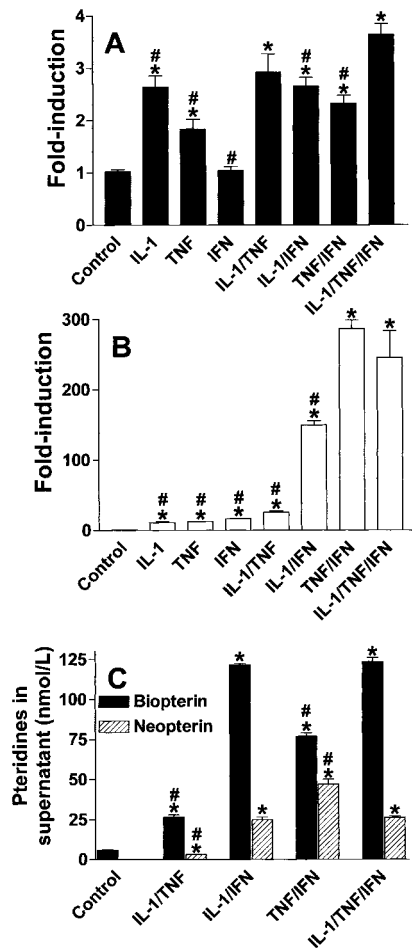


**Figure 1.** Transcriptional regulation of BH<sub>4</sub> synthesis in HCAECs. Time course of PTPS (A) and GTPCH (B) mRNA induction in HCAECs stimulated with IL-1 $\beta$ /TNF- $\alpha$ /IFN- $\gamma$  for various periods of time. Effect of simultaneous treatment with actinomycin D (Actino) and cycloheximide (Cyclo) on PTPS (C) and GTPCH (D) mRNA induction in HCAECs stimulated for 24 hours with IL-1 $\beta$ /TNF- $\alpha$ /IFN- $\gamma$ . Shown are mean  $\pm$  SEM from duplicate measurements of 3 or 4 independent experiments. \* $P < 0.0001$ , compared with control; # $P < 0.05$ , ## $P < 0.0001$ , compared with cells treated with IL-1 $\beta$ /TNF- $\alpha$ /IFN- $\gamma$  without inhibitor.

As a result, actinomycin D totally inhibited the induction of pteridine synthesis of IFN- $\gamma$ /TNF- $\alpha$ /IL-1 $\beta$ -treated cells (data not shown). Likewise, cycloheximide (0.8  $\mu$ g/mL) decreased PTPS and GTPCH mRNA of cytokine-stimulated HCAECs (Figure 1C and 1D) and reduced pteridine synthesis (data not shown).

We then investigated which cytokine was of major importance for the induction of PTPS mRNA. As shown in Figure 2A, IL-1 $\beta$  (and, to a lesser extent, TNF- $\alpha$ ) alone significantly increased PTPS mRNA levels, whereas IFN- $\gamma$  was ineffective. By comparison, single cytokines resulted in slight but significant induction of GTPCH mRNA levels (Figure 2B). TNF- $\alpha$ /IL-1 $\beta$  almost maximally induced PTPS mRNA, whereas GTPCH mRNA was still very low, a finding well in agreement with the previously identified importance of IFN- $\gamma$  for GTPCH induction.<sup>4</sup> Omission of TNF- $\alpha$  reduced PTPS mRNA levels by  $\approx 20\%$  and GTPCH mRNA levels by  $\approx 50\%$ , whereas omission of IL-1 $\beta$  did not diminish GTPCH mRNA abundance but decreased PTPS mRNA levels by  $\approx 40\%$  compared with fully stimulated HCAECs (Figure 2A and 2B).

Finally, we measured pteridines in culture supernatants to investigate the effect of gene expression changes on product formation. As shown in Figure 2C, only small amounts of BH<sub>4</sub> but no neopterin could be measured in supernatants of unstimulated cells, whereas both pteridines were significantly accumulated in supernatants of IFN- $\gamma$ /TNF- $\alpha$ /IL-1 $\beta$ -treated cells. IL-1 $\beta$  significantly contributed to total BH<sub>4</sub> synthesis but concomitantly limited the accumulation of GTPCH products (comparison of IFN- $\gamma$ /TNF- $\alpha$ /IL-1 $\beta$ -treated cells vs IFN- $\gamma$ /TNF- $\alpha$ -treated cells; Figure 2C). In other words, whereas stimulation with IFN- $\gamma$ /TNF- $\alpha$  resulted in a biopterin-neopterin ratio of  $1.7 \pm 0.1$ , treatment with IFN- $\gamma$ /TNF- $\alpha$ /IL-1 $\beta$  or IL-1 $\beta$ /TNF- $\alpha$  resulted in a biopterin-

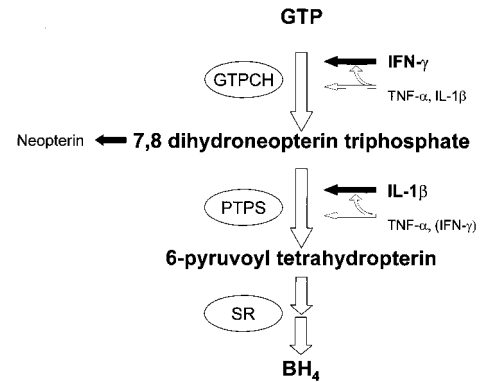


**Figure 2.** Differential effects of stimulatory cytokines on PTPS and GTPCH mRNA levels and pteridine secretion. Induction of mRNA levels of PTPS (A) and GTPCH (B) after activation of HCAECs with different cytokine combinations for 24 hours, as indicated. Shown are mean  $\pm$  SEM from duplicate measurements of 4 independent experiments. C, Measurement of pteridines in culture supernatants after 90 hours of cytokine treatment (mean  $\pm$  SD from 4 independent experiments). \* $P < 0.0001$ , compared with control; # $P < 0.002$ , compared with cells treated with IL-1 $\beta$ /TNF- $\alpha$ /IFN- $\gamma$ .

neopterin ratio of  $4.7 \pm 0.1$  or  $9.3 \pm 0.6$ , respectively. In contrast, IFN- $\gamma$  greatly increased total BH<sub>4</sub> synthesis (comparison of IFN- $\gamma$ /TNF- $\alpha$ /IL-1 $\beta$ -treated cells vs IL-1 $\beta$ /TNF- $\alpha$ -treated cells; Figure 2C) but significantly impaired the biopterin-neopterin ratio (ratio of  $1.7 \pm 0.1$  in IFN- $\gamma$ /TNF- $\alpha$ -treated cells vs  $9.3 \pm 0.6$  in IL-1 $\beta$ /TNF- $\alpha$ -treated cells).

### Discussion

In the present report, we show that BH<sub>4</sub> synthesis in HCAECs is regulated by inflammatory cytokines by altering both GTPCH and PTPS activity; the present and previous findings are summarized in Figure 3. Using quantitative PCR, we found a time-dependent, 4-fold upregulation of PTPS mRNA levels and an up to 300-fold upregulation of GTPCH mRNA levels in cytokine-stimulated cells compared with control cells. Earlier reports of potential regulation of PTPS have been equivocal.<sup>4</sup> However, the present finding of a 4-fold



**Figure 3.** Overview of de novo BH<sub>4</sub> synthesis in VECs. BH<sub>4</sub> is formed from GTP via GTPCH, leading to 7,8-dihydroneopterin triphosphate. PTPS then forms the tetrahydropterin intermediate that is reduced further to BH<sub>4</sub> in a 2-step reaction catalyzed by SR. BH<sub>4</sub> synthesis in VECs is regulated at 2 levels: (1) induction of GTPCH, mainly by IFN- $\gamma$  with less pronounced effects of TNF- $\alpha$  and IL-1 $\beta$ , and (2) induction of PTPS, mainly by IL-1 $\beta$  with a less pronounced effect of TNF- $\alpha$  and only little effect of IFN- $\gamma$ . Neopterin is derived from accumulating 7,8-dihydroneopterin triphosphate by phosphate elimination and oxidation. Abbreviations are as defined in text.

regulation of PTPS mRNA by cytokines in HCAECs is in agreement with our previous study that showed an  $\approx 10$ -fold enhanced PTPS mRNA abundance, as determined by conventional PCR, as well as a 3-fold-enhanced PTPS activity on cytokine stimulation in HUVECs.<sup>6</sup> Suppression of the cytokine-induced increase in PTPS mRNA and GTPCH mRNA expression by actinomycin D strongly indicates that the abundance of PTPS mRNA, GTPCH mRNA, and consequent BH<sub>4</sub> synthesis is mainly regulated at a transcriptional level in HCAECs on cytokine stimulation. This is further corroborated by studies with the protein synthesis inhibitor cycloheximide; although these data do not completely rule out an effect of cytokines on mRNA levels through posttranscriptional mechanisms, they suggest that protein-mediated stabilization of PTPS and GTPCH mRNA is unlikely to be of central importance. In contrast, IFN- $\gamma$ /TNF- $\alpha$ /IL-1 $\beta$  resulted in a 2.5-fold reduction of eNOS mRNA levels in HCAECs that was abolished by cycloheximide, whereas actinomycin D was ineffective (data not shown), confirming previous findings that showed a decrease of eNOS mRNA through induction of a protein that enhances eNOS mRNA degradation rather than through a direct influence on mRNA transcription<sup>9</sup> and that demonstrated that inflammatory cytokines regulate gene expression in VECs by  $>1$  mechanism.

Although cytokines induce only a modest increase in PTPS expression compared with their effect on GTPCH expression, increased PTPS expression significantly contributes to the total amount of BH<sub>4</sub> synthesized by HCAECs, and enhanced PTPS activity is required to maximize BH<sub>4</sub> synthesis while minimizing accumulation of the intermediary GTPCH product 7,8-dihydroneopterin triphosphate (see Figure 3). This can be explained by the fact that PTPS becomes rate-limiting for BH<sub>4</sub> synthesis on stimulation with a mixture of inflammatory cytokines, as evidenced by the significant accumulation of both biopterin and neopterin in supernatants of IFN- $\gamma$ /TNF- $\alpha$ /IL-1 $\beta$ -treated cells, whereas no neopterin ac-

accumulated in unstimulated cells. Comparison of the pteridine profiles (ie, the biopterin-neopterin ratio) in supernatants suggests that individual cytokines importantly influence the degree to which products of GTPCH accumulate. In HCAECs, IFN- $\gamma$  appeared as the single most important cytokine for total BH<sub>4</sub> synthesis. However, through its marked and predominant enhancement of GTPCH mRNA expression, IFN- $\gamma$  significantly impaired the biopterin-neopterin ratio because of a lack of sufficient PTPS activity. In contrast, addition of IL-1 $\beta$  to IFN- $\gamma$ /TNF- $\alpha$  greatly increased the biopterin-neopterin ratio in culture supernatants; ie, it decreased the relative accumulation of the GTPCH product neopterin. Because of its preferential enhancement of PTPS mRNA expression, therefore, IL-1 $\beta$  seems to play a crucial role with regard to optimization of BH<sub>4</sub> synthesis in VECs (Figure 3). As mentioned earlier, eNOS expression and consequently NO production are reduced in HCAECs under inflammatory conditions, suggesting that an imbalanced biopterin-neopterin ratio might not have significant effects on this system in inflamed VECs. However, an optimal biopterin-neopterin ratio might be critical for induced NOS (iNOS) by keeping inflammation-induced NO synthesis in vascular smooth muscle cells “coupled” under inflammatory conditions, with prevention of extensive formation of reactive oxygen intermediates as well as toxic peroxynitrite.<sup>3</sup>

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