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## Role of human GTP cyclohydrolase I and its regulatory protein in tetrahydrobiopterin metabolism

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■ **Abstract** *Objective* GTP cyclohydrolase I (GTPCH I) catalyzes the de novo biosynthesis of tetrahydrobiopterin (BH<sub>4</sub>), an essential cofactor of NO-synthase. The enzyme underlies negative feedback regulation by the end product BH<sub>4</sub>. This feedback inhibition is mediated through complex formation with the GTP cyclohydrolase I feedback regulatory protein (GFRP). To further classify the mechanism involved in the regulation of BH<sub>4</sub> synthesis, we measured expression of GTPCH I and GFRP in different human tissues. Furthermore, we looked for the influence of phenylalanine that is known to reverse BH<sub>4</sub>-mediated feedback inhibition of GTPCH I, and of immunostimulation with interferon  $\gamma$  on the expression of GTPCH I and GFRP. *Methods and results* Using RT-PCR and northern blot technique, coexpression of GFRP and GTPCH I could be demonstrated in a number of different tissues such as endothelial cells and peripheral blood cells. Following stimulation of human umbilical vein endothelial cells (HUVEC) with phenylalanine (1 mM), there was no change of GFRP mRNA. In contrast, the mRNA level of GTPCH I was significantly upregulated with a maximum after 6 hours ( $p = 0.04$ ). Incubation of HUVEC with interferon- $\gamma$  (100 U/ml) showed an increase of GTPCH I mRNA and a significant downregulation of GFRP mRNA after 24 hours ( $p = 0.03$ ). *Conclusion* This study shows for the first time the expression of GFRP in different human tissues. The biosynthesis of BH<sub>4</sub> is not only regulated on the substrate level but also through transcription of the interacting proteins. Phenylalanine stimulates the biosynthesis of BH<sub>4</sub> not only by reversing the negative feedback inhibition of GTPCH I but also by increasing the mRNA level of GTPCH I. Immunostimulation alters protein expression of GTPCH I and GFRP in a way that favors BH<sub>4</sub> synthesis.

■ **Key words** Endothelial function – free radicals – nitric oxide – tetrahydrobiopterin – vasoconstriction/dilation

### Introduction

6R-L-erythro-5,6,7,8-Tetrahydrobiopterin (BH<sub>4</sub>) is an essential cofactor required for catalytic activity of aromatic amino acid hydroxylases, which are key enzymes in the biosynthesis of several neurotransmitters and for the metabolism of phenylalanine in the liver (31). In

addition, BH<sub>4</sub> is an important cofactor for all three NO-synthase (NOS) isoforms (20, 32). Nitric oxide (NO) plays an important role in the regulation of vascular tone by mediating endothelium-dependent vasodilatation (22). Furthermore, NO is a potent inhibitor of platelet and leukocyte adhesion to the vascular wall and inhibits smooth muscle cell proliferation (22).

There is a positive correlation between the availability of BH<sub>4</sub> and the biosynthesis of NO in endothelial cells and smooth muscle cells (1). The role of BH<sub>4</sub> in the biosynthesis of NO is not completely understood. Current experimental evidence suggests that it may work both as an allosteric and a redox cofactor and that it facilitates the binding of L-arginine to NO-synthase (20). A decreased availability of BH<sub>4</sub> results in a dysfunction of NO-synthase, shifting the balance between the production of protective NO and deleterious oxygen-derived free radicals towards the latter (1). This imbalance is important in understanding the mechanisms of endothelial dysfunction and oxidative vascular injury as generally described in vascular pathology (2, 3, 11, 12, 14, 22, 24, 28).

BH<sub>4</sub> is synthesized in endothelial cells via both the salvage pathway from sepiapterin and the de novo pathway from guanosine triphosphate (GTP) by the sequential action of three enzymes: GTP cyclohydrolase I (GTPCH I), 6-pyruvoyltetrahydrobiopterin synthase and sepiapterin reductase (15, 25, 32). GTPCH I catalyzes the initial and rate limiting reaction of the de novo biosynthesis of BH<sub>4</sub>. The activity of GTPCH I is regulated at transcriptional and substrate levels (8, 9, 29) and is sensitive to end-product feedback inhibition by BH<sub>4</sub> (7). BH<sub>4</sub> induces the formation of a complex between GTPCH I and the GTP cyclohydrolase I feedback regulatory protein (GFRP). L-phenylalanine specifically reverses the tetrahydrobiopterin-dependent inhibition of GTPCH I (7). This may explain the high plasma BH<sub>4</sub> concentrations observed in patients with hyperphenylalaninemia caused by phenylalanine hydroxylase deficiency (5, 17, 18).

GFRP has been first described by Harada et al. in crude rat liver extracts. The cDNA clone from a rat liver cDNA library encodes a protein of 84 amino acids (21). GFRP was found to consist of a pentamer of 9.5-kDa identical subunits (34). Two molecules of a pentameric GFRP associate with one molecule of GTPCH I (34). BH<sub>4</sub> and GTP are required for the complex formation (35). Northern blot analysis indicated the presence of three mRNA transcripts with the length of 0.8 kb, 1.4 kb and 6.0 kb in most rat tissues. High expression was found in liver and kidney, lower expression in heart, brain, lung and testes, and very low expression in muscle and spleen (21).

The presence of the protein GFRP in human cells has only been described in keratinocytes and recently in myelomonocytoma (THP-1) cells and HUVEC (26, 31). We, therefore, investigated the expression of GFRP and GTPCH I mRNA in different human tissues with the main focus on endothelial cells by RT-PCR and northern blot technique. To obtain insight into the regulation of GFRP and GTPCH I in humans, we investigated the influence of phenylalanine and interferon- $\gamma$  on the mRNA synthesis of GFRP and GTPCH I in HUVEC with semi-quantitative RT-PCR.

## Methods

### ■ Cell culture

Human umbilical vein endothelial cells (HUVEC) were prepared from human umbilical cord after the method of Jaffe et al. (13). HUVECs were maintained in Endothelial Cell Growth Medium with supplement pack (10% fetal calf serum, endothelial cell growth supplement and antibiotics) from PromoCell. For the experiments, HUVEC were used at passage level 3 and after they had grown to a confluent monolayer in plastic culture dishes. For each experiment the cells were descended from one umbilical cord. Twenty-four hours before the experiment, the medium was changed to Endothelial Cell Basal Medium with 2% FCS. HUVEC were incubated with phenylalanine (1 mM) and interferon- $\gamma$  (100 U/ml) for 24 hours. For RNA preparation the cells were lysed after 0, 1, 3, 6, 12 and 24 hours.

### ■ RNA isolation

The RNA from HUVEC was extracted with the RNeasy Mini Kit (Qiagen). The RNA from human blood was isolated by RNeasy Blood Mini Kit (Qiagen). Mononuclear cells from human blood were isolated by Ficoll-Paque<sup>TM</sup> PLUS (Amersham) and the RNA was isolated after the protocol of the RNeasy Blood Mini Kit (Qiagen). The RNA was stored at -70 °C.

### ■ RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed. First strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase. The cDNA was amplified by PCR with synthetic gene-specific primers. The used primers were: GTPCH I primer I: 5'-CAGGAGACCATCTCAGATGTC-3'; primer II: 5'-TTCTTCTCCCTTCCCAGGCC-3'; GFRP primer I: 5'-CACCATGCCCTACCTGCTCA-3'; primer II: 5'-CCTTGTGCAGACACCACACC-3'. The PCR conditions were 1 min of denaturation at 94 °C, 1 min of annealing at 60 °C and 1 min of extension at 72 °C for 35 cycles for all proteins. To ensure that equal amounts of reverse-transcribed RNA were added to the PCR, we subjected glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to amplification in parallel as a reference with the primers: primer I: 5'-TGAAGGTCGGAGT-CAACGGATTTG-3'; primer II: 5'-CATGTGGGCCAT-GAGGTCCACCAC-3'. With 30 cycles for GTPCH I and GAPDH and 29 cycles for GFRP, amplification was in a linear range. The PCR products were separated by electrophoresis on a 1.2% agarose gel containing ethidium

bromide and were visualized by ultraviolet light-induced fluorescence. The PCR resulted in single products for GFRP (254 bp) and GTPCH I (720 bp). The identity of the PCR products was confirmed by sequencing of the product.

### Northern blot

For each lane of the northern blot, 20 µg of total RNA was used. The PCR fragment was labeled with [<sup>32</sup>P]dCTP with the random prime labelling system from Amersham Pharmacia Biotech. The northern blot was hybridized after the protocol of ExpressHyb™ Hybridization Solution (Clontech). The recommended final DNA probe concentration was 2 × 10<sup>6</sup> cpm/ml. The membranes were prehybridized with 5 ml ExpressHyb Solution at 68 °C for 30 min. The radiolabeled probe was added and incubated at 68 °C for 1 hour. After the incubation the blot was washed and exposed to X-ray film at -70 °C.

The human 12-lane Multiple Tissue Northern (MTN)<sup>TM</sup> Blot used in this study was from Clontech and hybridized following the same protocol. The X-ray tracings were recorded with a Photoimager (Fluor-STM MultiImager from BIO-RAD) and quantified using Multi-analyst software. To ensure that equal amounts of RNA were loaded, each northern blot was hybridized with a PCR fragment from GAPDH as a reference.

### Materials

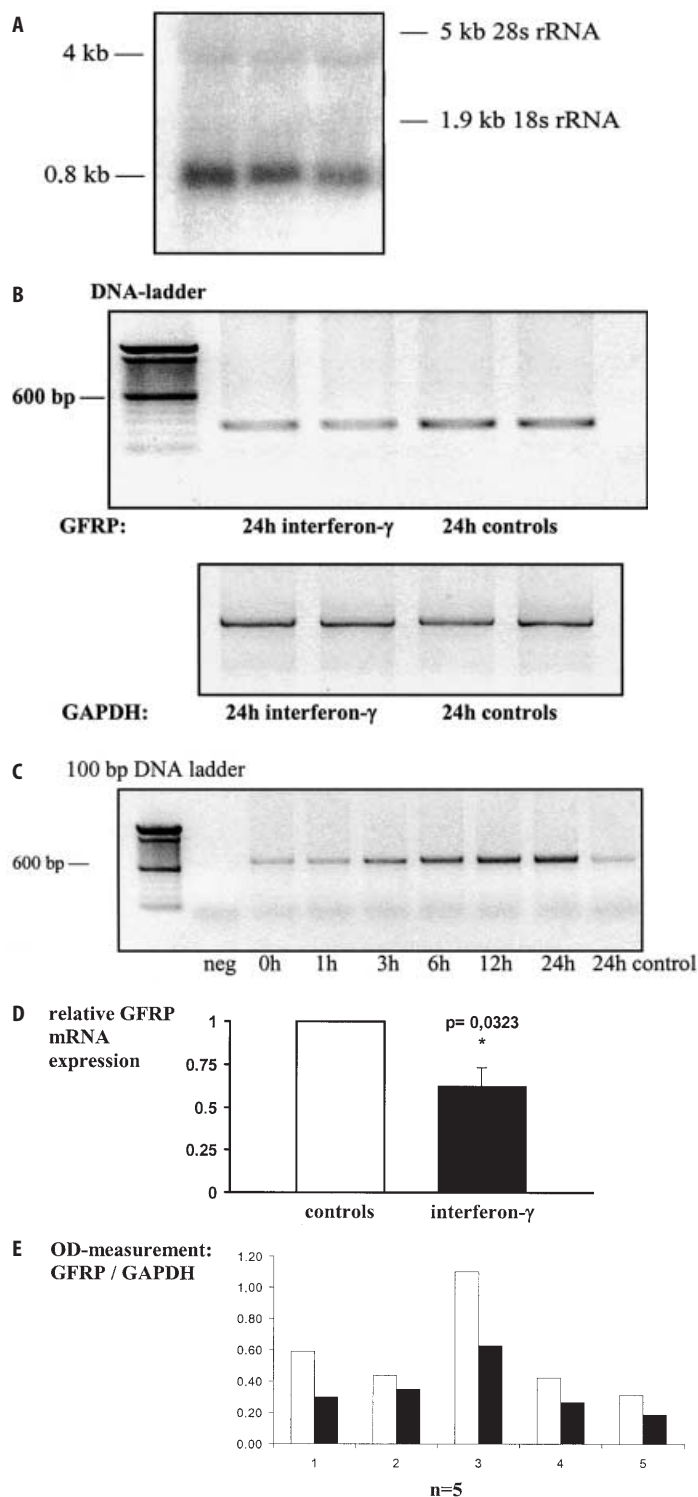
L-Phenylalanine was obtained from ICN Biochemicals, human interferon-γ (100 000 U/ml) from Boehringer Mannheim and the human 12-Lane Multiple Tissue Northern (MTN<sup>TM</sup>) Blot from Clontech Laboratories. All other chemicals were purchased from Sigma, Roth and Merck.

## Results

### Expression of GFRP and GTPCH mRNA

In all investigated human tissues and cells an expression of GFRP mRNA and GTPCH I mRNA could be demon-

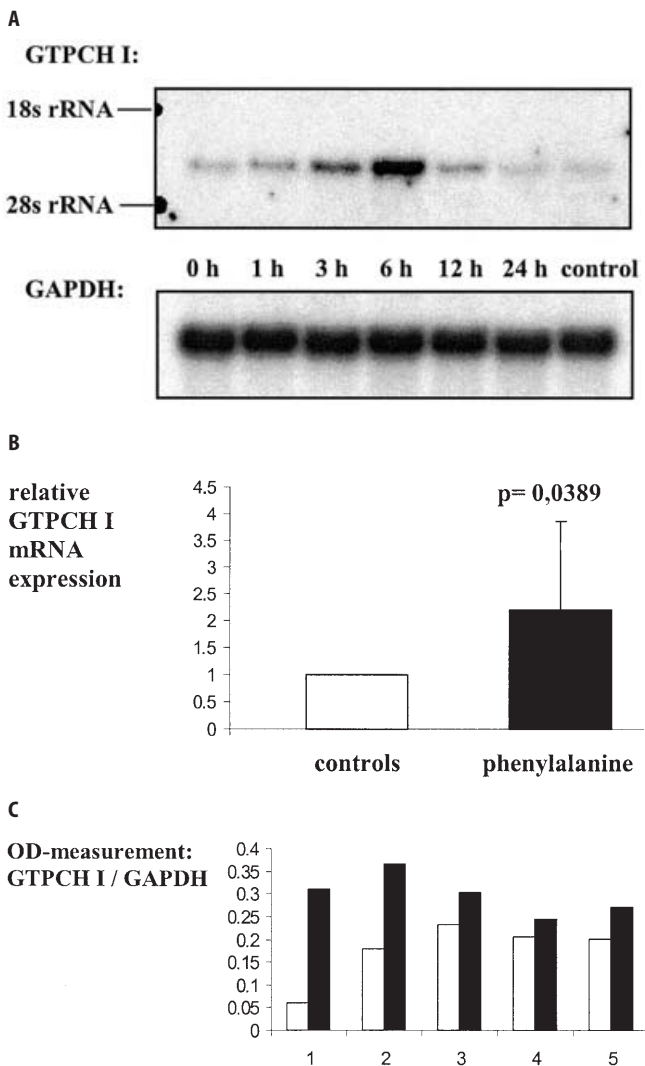
**Fig. 1** **A** Northern blot analysis of total RNA of human umbilical vein endothelial cells. Hybridization was performed with a [<sup>32</sup>P]dCTP-labeled PCR fragment of GFRP. Two mRNA species of GFRP were detected in all samples with a size of 4 kb and 0.8 kb. Lane 1–3: 20 µg total RNA from HUVEC from three individuals. **B** Gel electrophoresis of RT-PCR analysis of GFRP-mRNA in HUVEC (n = 2): change in GFRP-mRNA level following treatment with interferon-γ (100 U/ml) for 24 hours. The RT-PCR shows a downregulation of the mRNA level after 24 hours. The results with GAPDH primers are shown for comparison. **C** Induction of GTPCH I mRNA after treat-



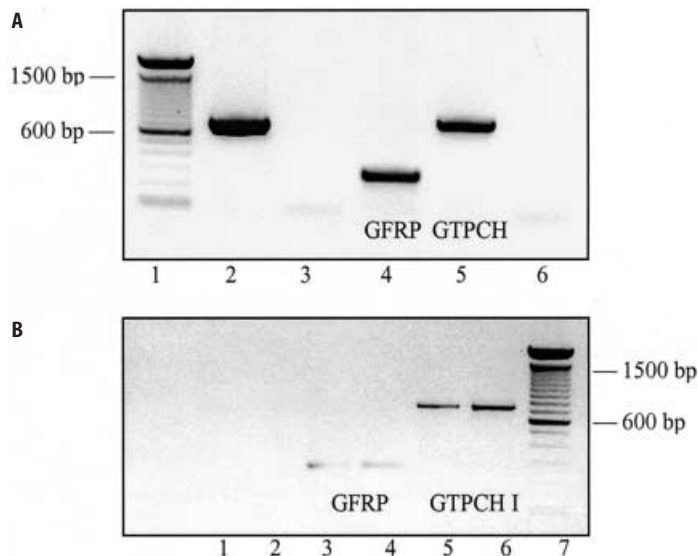
ment of HUVEC with interferon-γ (100 U/ml; n = 5). The cells were lysed at time points 0, 1, 3, 6, 12, 24 hours after incubation. Shown is the gel electrophoresis of the RT-PCR. Negative control (neg) and 24 h control are without interferon-γ. **D** Downregulation of GFRP mRNA after treatment of HUVEC with interferon-γ (100 U/ml) for 24 hours in RT-PCR. The mRNA decreases up to 37% as compared to control (n = 5; p = 0.03). **E** RT-PCR analysis of GFRP-mRNA in HUVEC after treatment with interferon-γ (100 U/ml) for 24 hours (n = 5). Controls: open bars, interferon-γ treatment: full bars.

strated. In HUVEC, GFRP mRNA was detected by RT-PCR (Fig. 1B). Northern blot analysis showed two mRNA species with a length of 0.8 kb and 4 kb (Fig. 1A). GTPCH I mRNA was detected in HUVEC by northern blot analysis (Fig. 2A) and RT-PCR (Fig. 1C). Similarly, RT-PCR analysis from human blood isolated leukocytes, lymphocytes and monocytes showed mRNA coexpression of both proteins (Fig. 3A and B).

GFRP mRNA expression was investigated in a human 12-lane Multiple Tissue Northern (MTN)<sup>TM</sup> Blot (Clontech). Each lane contains 1 µg Poly-A<sup>+</sup>-RNA from the



**Fig. 2** **A** Northern blot analysis of GTPCH I mRNA in HUVEC following treatment with phenylalanine (1 mM; n = 5). The northern blot shows an increase of the mRNA level after 6 hours. The results of the hybridization with GAPDH are shown for comparison. **B** Induction of GTPCH I mRNA after treatment with phenylalanine (1 mM) for 6 hours. The mRNA increases up to 2.2 fold as compared to control (n = 5; p = 0.04). **C** Northern blot analysis of the induction of the GTPCH I mRNA in HUVEC after treatment with phenylalanine for 6 hours (n = 5). Controls: open bars, phenylalanine treatment: full bars



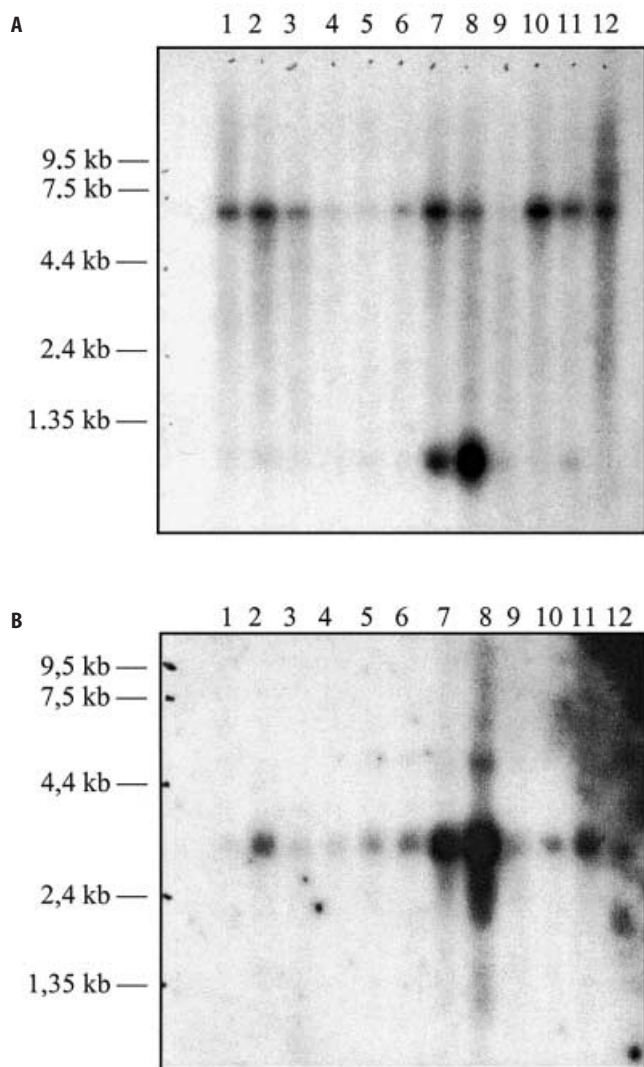
**Fig. 3** mRNA expression of GFRP and GTPCH I in leukocytes (**A**), lymphocytes and monocytes (**B**) from human blood. The RNA was prepared and assayed by RT-PCR. **A** lane 1: 100 bp DNA ladder, 2: positive control, 3: negative control, 4: PCR with GFRP primer, 5: PCR with GTPCH I primer, 6: negative control. **B** lanes 1 and 2: negative control, 3 and 4: PCR with GFRP primer from samples of different individuals, 5 and 6: PCR with GTPCH I primer from samples of different individuals, 7: 100 bp DNA ladder

specific tissue. Hybridization with the GFRP probe detected two mRNA species with a length of 6.5 and 0.8 kb in most human tissues (Fig. 4A). The levels of the 0.8 kb mRNA were high in liver and kidney. The other tissues showed only a low detection of the 0.8 kb mRNA. The expression of the 6.5 kb mRNA was high in placenta, kidney, peripheral blood leukocytes, heart, and low in tissue from brain, liver, lung and skeletal muscle. Very low expression was found in tissue from spleen, thymus, colon and small intestine.

The same human 12-lane Multiple Tissue Northern (MTN)<sup>TM</sup> Blot was used for characterization of GTPCH I (Fig. 4B). The hybridization showed two mRNA species with a length of 3.6 kb and 4.6 kb in liver and kidney. The other tissues showed only the 3.6 kb mRNA species. The highest levels of the 3.6 kb mRNA were found in liver and kidney; lower levels were found in lung, heart, peripheral blood leukocytes, spleen, thymus and placenta and very low levels in colon, skeletal muscle, brain and small intestine.

#### ■ Stimulation with phenylalanine and interferon $\gamma$

The effect of phenylalanine and interferon- $\gamma$  on the mRNA level of the GFRP and the GTPCH I was investigated. HUVEC (n = 5) were incubated with phenylalanine and interferon- $\gamma$  and the cells were investigated at 0, 1, 3, 6, 12 and 24 h. The RNA was prepared and assayed



**Fig. 4** Detection of GFRP and GTPCH mRNA in various human tissues. A nylon membrane containing polyadenylated RNA isolated from various human tissues (1  $\mu$ g/lane) was hybridized with a [ $^{32}$ P]dCTP-labeled PCR-generated DNA fragment of GFRP and GTPCH I, respectively. Lane 1: brain, 2: heart, 3: skeletal muscle, 4: colon, 5: thymus, 6: spleen, 7: kidney, 8: liver, 9: small intestine, 10: placenta, 11: lung, 12: peripheral blood leukocytes. **A** GFRP: In most human tissues, two mRNA species with the length of 6.5 and 0.8 kb were detected. **B** GTPCH: The hybridization showed two mRNA species with the length of 3.6 kb and 4.6 kb in liver and kidney. The other investigated tissues showed only the 3.6 kb mRNA species

by RT-PCR. From each probe, a RT-PCR with specific primers for GAPDH was made for comparison.

Stimulation of HUVEC with interferon- $\gamma$  (100 U/ml) showed a significant downregulation of GFRP mRNA after 24 h ( $p = 0.03$ ; Fig. 1D) with reduction of 40% as compared to the non-stimulated control in cells from five different HUVEC preparations (Fig. 1E). In contrast, the GTPCH I mRNA level was significantly increased by 2.4-fold following stimulation with Interferon- $\gamma$  after 24 h ( $n = 5$ ;  $p = 0.04$ ; Fig. 1C).

Stimulation of HUVEC with phenylalanine (1 mM) induced no change in GFRP mRNA level over 24 hours. In contrast the mRNA level of the GTPCH I was significantly increased by 2.2-fold with a maximum after 6 h ( $p = 0.04$ ;  $n = 5$ ; Fig. 2A–C). Figure 2 shows the effect of phenylalanine on the induction of GTPCH I mRNA level.

## Discussion

In the present study, the mRNA expression of GFRP and GTPCH I was investigated in different human tissues focussing on endothelial cells. It was found that both are expressed in all investigated tissues. In endothelial cells, GTPCH I catalyzes the initial and rate-limiting reaction for the de novo synthesis of BH $_4$ , a critical cofactor for the biosynthesis of EDRF. The activity of GTPCH I is both inhibited by BH $_4$  and stimulated by phenylalanine through complex formation with GFRP (7). The expression of GTPCH I and GFRP mRNA in human endothelial cells implies that a similar regulatory mechanism may exist in endothelial cells regarding the biosynthesis of BH $_4$ .

Expression of GFRP and GTPCH I mRNA could also be demonstrated for the first time in leukocytes, lymphocytes and monocytes from peripheral human blood. BH $_4$  is required as a cofactor for the biosynthesis of NO in granulocytes and macrophages. In hematopoietic progenitor cells and activated T cells, BH $_4$  acts as a positive growth regulator which increases the proliferation rate of erythroid cells and enhances the clonal expansion of T cells (27).

Hybridization with the GFRP probe detected a similar expression pattern in the different tissues as found previously in rat tissue (21). In this study, three mRNA species with a length of 0.8 kb, 1.4 kb and 6.0 kb have been described. The minor bands at 1.4 and 6 kb were interpreted as to represent incompletely spliced RNAs. The 0.8 kb mRNA was also high in rat liver and kidney. Lower levels were found in heart, brain, lung, and testis of rats. Very low level were detected in skeletal muscle and spleen (21).

Hybridization of the multiple tissue northern blot with the GTPCH I probe detected a mRNA species with the length of 3.6 kb in all investigated tissues. In liver and kidney, hybridization identified a second mRNA of 4.6 kb. The 3.6 kb mRNA has been previously identified in the human T-cell line HUT and in the human liver-cell line HuH7 (6). In contrast, the size of human GTH I mRNA has been shown by cloning to be 2.9 kb rather than 3.6 kb by others (5). In our study, mRNA of GTPCH I was detected in all investigated human tissues. Concomitant to the level of GFRP mRNA, the expression of GTPCH I mRNA was high in liver, kidney, lung and heart.

The biosynthesis of BH<sub>4</sub> may be of pathophysiological importance in atherosclerosis which is a local inflammatory process of the vessel wall. We therefore investigated the influence of the immunostimulator interferon- $\gamma$  on the expression of GTPCH I and GFRP in human umbilical vein endothelial cells (HUVEC).

Treatment with interferon- $\gamma$  increased the expression of GTPCH I as has been previously reported (10, 16, 19). In addition, interferon- $\gamma$  decreased the mRNA level of GFRP significantly by 40% with a maximum after 24 hours. The result of such a dual regulation would be an increased capacity for the synthesis of BH<sub>4</sub> by increasing the availability of the rate limiting enzyme and by decreasing negative end product-mediated feedback regulation. Our findings are partially supported by observations of Werner et al. (31) describing a significant suppression of GFRP mRNA in LPS-treated and in interferon- $\gamma$  plus LPS-treated HUVEC. In contrast to our study, interferon- $\gamma$  alone did not significantly suppress GFRP mRNA. This may be explained by the shorter exposition to interferon- $\gamma$  for 6 h in contrast to the 24 h treatment of our study. Additionally, our findings are somewhat different to observations by Xie et al. (33) who investigated mRNA expression of GFRP in smooth muscle cells of rat aorta and described an upregulation of GFRP mRNA of about 2-fold after 24 h. The discrepancy may reflect differences of BH<sub>4</sub> metabolism in different cell types and species.

Phenylalanine has been found to reverse the negative feedback inhibition of GTPCH I by BH<sub>4</sub> in rat liver. For this reason we investigated the influence of phenylala-

nine (1 mM) on the mRNA levels of GFRP and GTPCH I in HUVEC. Orally given phenylalanine enhances the serum concentration of phenylalanine and neopterin, a degradation product of the BH<sub>4</sub> precursor, 7,8-dihydro-neopterin triphosphate (4). In patients with phenylketonuria, increased concentrations of BH<sub>4</sub> were found (18).

Phenylalanine had no influence on the mRNA level of GFRP; however, the GTPCH I mRNA level was significantly upregulated with a maximum after 6 hours. This observation suggests that enhanced levels of phenylalanine induce the transcription of GTPCH I. Thus, phenylalanine enhances the BH<sub>4</sub> concentration not only through reversing the negative feedback inhibition of GTPCH I but also through induction of the GTPCH I-mediated biosynthesis. However, the underlying mechanisms by which phenylalanine alters transcription are to date unknown.

In summary, we could show an expression of GFRP and GTPCH I mRNA in all investigated human cells and tissues. The expression of GTPCH I was upregulated by application of phenylalanine. Interferon- $\gamma$  upregulated the expression of GTPCH I mRNA and downregulated the expression GFRP mRNA. These results put further focus on the significance of BH<sub>4</sub> metabolism. In future studies, it needs to be determined if gene expression of the two enzymes is reflected at the functional level.

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