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Analysis of the effect of tetrahydrobiopterin on PAH gene expression in hepatoma cells

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Abstract Tetrahydrobiopterin (BH4)-responsive phenylalanine hydroxylase (PAH) deficiency is a recently recognized variant of phenylketonuria, with a probable multifactorial molecular basis. In this study we have investigated the effect of BH4 on PAH gene expression in human hepatoma. Our results show that increased BH4 levels result in an enhancement of PAH activity and PAH protein, due to longer turnover rates, while PAH mRNA levels remain unchanged. This was confirmed for mutant PAH proteins (A309V, V388M and Y414C) associated to in vivo BH4 responsiveness, validating previous studies. We can conclude that there is no effect of the cofactor on PAH gene transcription, probably being the chemical chaperone effect of BH4 stabilizing mutant PAH proteins the major underlying mechanism of the response.
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1. Introduction

Tetrahydrobiopterin ((6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin, BH_4) is the natural cofactor of phenylalanine hydroxylase (PAH), an hepatic enzyme that catalyzes the hydroxylation of L-Phe to L-Tyr and which is defective in the autosomal recessive disorder phenylketonuria (PKU) [1]. The accumulation of L-Phe in body fluids can be effectively avoided by classical dietary therapy preventing neurological damage. In addition, supplementation with high doses of the cofactor can be an effective therapy for a group of PKU patients [2]. Many studies conducted worldwide have recently demonstrated that there is a high proportion of patients (60–70%), who respond positively to a BH4 loading test [3–5] and several reports have begun to appear proving the efficacy of long-term treatment with BH4 [6–9].

Examination of the BH4-responsive patients' genotypes shows a high genetic heterogeneity, although some mutations have been repeatedly associated to the response. In vitro expression data have shown that only a few of these mutations correspond to K_M mutants having a slightly decreased affinity for BH4, and for other mutants stabilization by BH4 was observed [10,11]. In vivo, in the liver of patients with BH4 deficiencies, reduced PAH activity and/or protein have been reported [12,13]. This already suggested that BH4 concentration may be an important regulatory factor in maintaining the steady-state levels of PAH. Similar results were also observed in transgenic mice deficient in cofactor biosynthesis [14,15]. In transgenic mice with a complete or partial deficiency in 6-pyruvoyltetrahydropterin synthase (PTPS), the rate of PAH activity and protein levels increased with BH4 content without affecting PAH mRNA levels, apparently ruling out an effect on gene expression [15]. However, for inducible nitric oxide synthase (iNOS), which also requires BH4 as essential cofactor, a post-transcriptional stabilization of iNOS mRNA by BH4 has been described in several cell types [16,17].

In this study we sought out to analyze the effect of BH4 levels on PAH gene expression in human hepatoma cells, where PAH and the enzymes responsible for the BH4 biosynthetic pathway are naturally expressed. Precursors and inhibitors of the intracellular synthesis pathway, which involve three enzymes (GTP cyclohydrolase I, GTPCH-1, PTPS and sepiapterin reductase, SR) [18,19], were used to modulate BH4 levels.

The results of increasing BH4 levels in hepatoma cells discard an effect of the cofactor on PAH gene transcription, and confirm a chaperon-like effect of BH4 lowering PAH degradation rate. The stabilization effect was also demonstrated for three mutant PAH proteins associated to BH4 response and which were tagged with a FLAG epitope and transfected into hepatoma cells for measurement of protein steady state levels in the presence of increased levels of BH4.

2. Materials and methods

2.1. Materials

Sepiapterin was obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). Actinomycin D, puromycin, dicumarol and 2,4-diamino-6-hydroxypyrimidine (DAHP) were from Sigma–Aldrich. Cell culture medium and fetal calf serum (FCS) were from Gibco-BRL. Monoclonal antibody anti PAH (PH8) was from Pharmingen. Anti-FLAG M2 antibody was from Sigma. The secondary antibody (antimouse Ig conjugated with horseradish peroxidase) was from Santa Cruz. The Jetpei transfection reagent was obtained from Polyplus transfections.

2.2. Cell culture and transfection

Human hepatoma cells Hep3B were cultured under standard conditions in minimum essential medium supplemented with 1% glutamine, 10%FCS and antibiotics. Cells were supplemented with 100 μM sepiapterin or 100 μM dicumarol or 10 mM DAHP for the times indicated. For transient expression of PAH-FLAG constructs, 4×10^6 cells were plated in T-75 flasks and transfected with Jetpei following the

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Abbreviations: BH4, (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin; PAH, phenylalanine hydroxylase; PKU, phenylketonuria; DAHP, 2,4-diamino-6-hydroxypyrimidine

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manufacturer's recommendations. After 5 h incubation the Jetpei–DNA mixture was removed and replaced with fresh medium supplemented or not with the various reagents. PAH-FLAG proteins were analyzed after 48 h.

2.3. Determination of intracellular biopterin

Hep3B cells treated with the indicated agents were harvested by trypsinization and washed with phosphate-buffered saline. Pellets were resuspended in 100 mM Na-HEPES (pH 7.0) and lysed by freeze–thawing. After centrifugation to remove cell debris, the supernatant (total cell extract) was used for fluorimetric determination of total biopterin [3].

2.4. Determination of PAH activity

After treatment with the various reagents for 48 h, PAH activity in cell extracts obtained as described above was measured for 30 min at 25 °C as conversion of L-Phe to L-Tyr [10] and after elimination of aminoacids using Ultrafree-MC 10000 NMWL filters. The amount of L-Tyr formed was measured by HPLC and fluorimetric detection.

2.5. Protein analysis

Wild-type PAH protein or transfected PAH-FLAG proteins in Hep3B cells were detected by Western blotting using commercial monoclonal antibodies (anti PAH Ph8 or anti-FLAG M2, respectively), anti-mouse-Ig hoseradish peroxidase conjugate and the enhanced chemiluminiscence detection system ECL (Amersham). For determination of protein turnover, after treatment with the various reagents for 48 h, protein synthesis was stopped by addition of 10 μg/ml puromycin to the culture medium and cells were harvested at defined time points up to 10 h and analyzed by Western blotting. Relative protein amounts were determined by densitometric analysis and expressed as percentage of residual protein.

2.6. mRNA analysis

Total cellular RNA was isolated using the SV Total RNA Isolation Kit (Promega). For relative PAH mRNA quantification, the Taqman Gene Expression assay Hs00609359 (Applied Biosystems) was used. RNA was retrotranscribed using the Archive kit and PCR amplified with the Taqman Universal PCR Master Mix, all from Applied Biosystems. The real-time PCR and analysis were performed in an ABIPRISM 7900HT Genetic Analyzer. Amplification efficiency and sample to sample variation were normalised by monitoring glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA or 18S rRNA.

2.7. Cloning into pFLAG vector

PAH cDNA was amplified from pMAL-PAH expression vector [20] using primers 5′-TTTTGCGGCCGCGATGTCCACTGCGGTCCTGG-3′ and 5′-AAAAGTCGACGGCTTTACTTTATTTTCTGGAG-3′, designed with *Sal*I and *Not*I restriction sites at their 5′ ends, respectively, to allow directed cloning into the pFLAG-CMV vector (Sigma). The in-frame fusion of amino-terminal FLAG peptide with the PAH protein was confirmed by sequencing performed with BigDye Terminator v.3.1 mix (Applied Biosystems) and analyzed by capillary electrophoresis on an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). Mutations were introduced into the wild-type construct using the Quickchange mutagenesis kit (Stratagene) and confirmed by sequencing analysis.

3. Results

Human hepatoma cells were used as cellular model to determine the effect of BH4 on PAH gene expression. Sepiapterin was used as precursor of the intracellular BH4 biosynthetic pathway and dicumarol or DAHP as inhibitors of enzymes involved in BH4 synthesis (of SR and GTPCH-1, respectively) to reduce BH4 levels.

Pterin analysis in Hep3B cells cultured in standard conditions revealed levels of 15.4 pmol/mg of total biopterin. After 48 h supplementation with 100 μM sepiapterin in the culture medium, biopterin levels increased to 4601 ± 1633 pmol/mg, while treatment with dicumarol (100 μM) or DAHP (10 mM) reduced total biopterin to undetectable levels (Fig. 1A). A similar increase or decrease in biopterin levels after supplementation

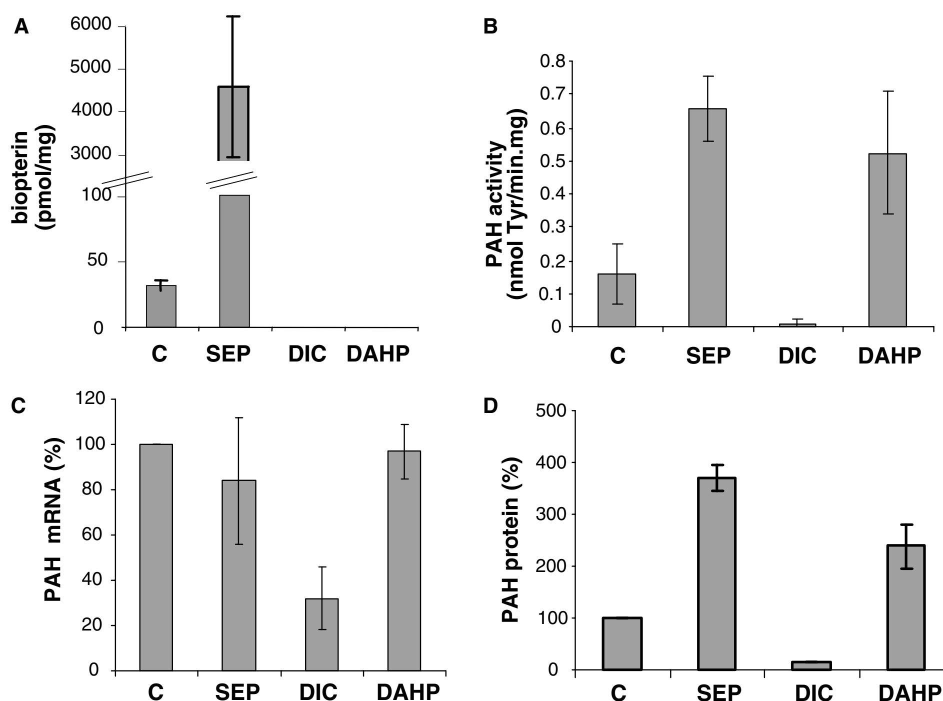

Fig. 1. Effect of precursors and inhibitors of the BH4 biosynthetic pathway on: (A) biopterin content; (B) PAH activity; (C) PAH mRNA and (D) PAH protein in hepatoma cells. After supplementation with sepiapterin (100 μM), dicumarol (100 μM) or DAHP (10 mM) for 48 h, total biopterin, PAH activity, PAH mRNA and PAH protein content were measured in cellular extracts as described in Section 2. The data are the means ± S.D. of at least three experiments. SEP, sepiapterin; BH4, precursor; DIC, dicumarol; SR, inhibitor; DAHP, GTPCH-1 inhibitor.

with sepiapterin or dicumarol/DAHP, respectively, was confirmed in all subsequent experiments regarding PAH activity, protein or mRNA.

Supplementation with sepiapterin produced a ∼4-fold enhancement in PAH activity in hepatoma cells. Treatment with dicumarol abolished PAH activity completely while DAHP unexpectedly increased it (Fig. 1B).

The potentiation of PAH activity with sepiapterin could be due to BH4 playing a regulatory role at the level of protein or mRNA expression. To test this hypothesis, we first examined PAH mRNA levels in hepatoma cells cultured in standard medium alone or supplemented for 48 h with sepiapterin, dicumarol or DAHP. Quantitation of PAH mRNA levels revealed no significant differences between culture conditions, with only a slight reduction in mRNA levels in dicumarol treated cells (Fig. 1C). However, direct observation of the cells with dicumarol before harvesting showed generalised cell death which could explain this result.

The influence of BH4 on PAH activity observed in sepiapterin treated cells could thus be ascribed to the post-transcriptional level. We next examined PAH protein levels by Western blot analysis, which revealed that in sepiapterin-supplemented cells PAH protein was strongly augmented, correlating with the increase in PAH activity. In dicumarol treated cells no PAH was detectable, while with DAHP, PAH protein levels were increased correlating with the results obtained for PAH activity (Fig. 1D).

To determine whether BH4 levels were affecting steady-state protein turnover and taking into account previous evidence of a stabilization effect of BH4 on PAH proteins [10,11,15], we measured PAH protein half-life in hepatoma after treatment with the different compounds. Protein synthesis was stopped with translation inhibitor puromycin (10 μg/ml) and protein amounts were determined by Western blot at defined time points up to 10 h (Fig. 2). PAH protein half-life in hepatoma was estimated at 9.4 h, in line with previous reports in an in vitro cell-free synthesis system [10,11]. Treatment with sepiapterin increased PAH half-life ∼3-fold, confirming that BH4 may exert a protective effect preventing degradation of PAH protein.

To validate the results obtained previously in a cell-free synthesis system [10,11] and to provide a rationale for BH4 responsiveness in PKU, the effect of BH4 on previously studied mutant PAH proteins (A309V, V388M and Y414C) was reassessed in hepatoma. For this purpose, wild-type and mutant PAH constructs tagged with a FLAG epitope were engineered in the expression vector pFLAG-CMV. The A309V, V388M and Y414C mutations had been shown in an in vitro expression system to be stabilized by BH4 [10,11].

Transfection of the wild-type construct in hepatoma cells resulted in the expression of PAH-FLAG protein detected by Western blot analysis using anti-FLAG antibody, thus precluding the detection of endogenous PAH. Estimation of PAH-FLAG protein half-life was 9.4 h, the same as that obtained with endogenous PAH, showing that the FLAG peptide does not alter significantly the properties of the PAH protein.

Transfection of the mutant constructs A309V, V388M and Y414C revealed that the steady-state amount of protein is somewhat reduced compared to wild-type (Fig. 3), in correlation with what had already been reported by expression analysis in COS cells, indicative of folding defects [20]. Although in this system we have to take into account the presence of endogenous PAH protein which could be forming oligomers with the transfected mutant proteins stabilising them, we sought to find out whether steady-state levels of mutant proteins were increased with sepiapterin. As shown in Fig. 3, in sepiapterin treated cells, the amount of all three mutant proteins is effectively increased although this effect is minimal for wild-type PAH-FLAG protein.

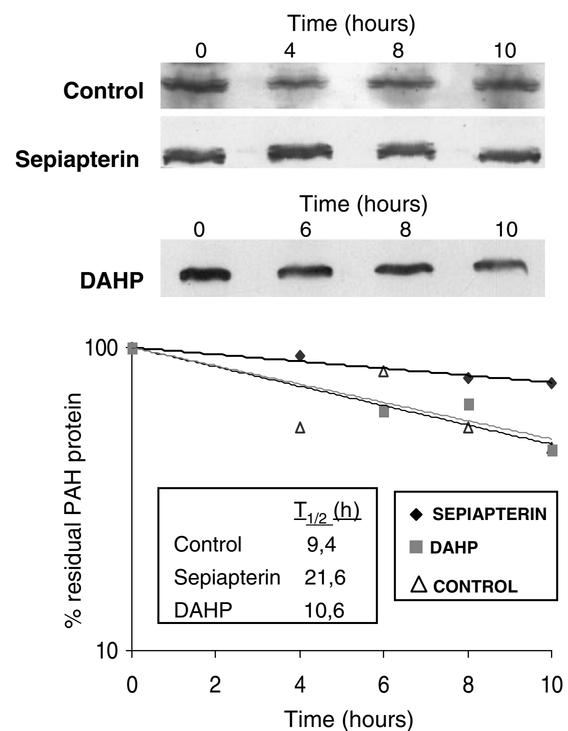

Fig. 2. Analysis of PAH protein turnover in hepatoma cells. After incubation with sepiapterin for 48 h, translation was stopped with puromycin (10 μg/ml) and PAH protein in cells harvested up to 10 h was analysed by Western blot analysis (upper panel). The lower panel shows the representation of the results as a semilogarithmic plot of relative PAH protein obtained after densitometric analysis of Western blot autoradiograms. Shown is the mean of two independent experiments. The inner box shows the estimated half-lives of PAH protein in each condition.

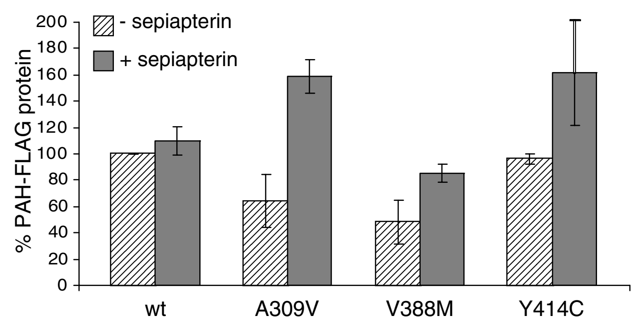

Fig. 3. Steady-state levels of wild-type and mutant PAH-FLAG proteins transiently expressed in hepatoma cells in the presence or not of sepiapterin as BH4 precursor. PAH-FLAG proteins were detected in cellular extracts using anti-FLAG antibodies and quantified by densitometric analysis. The data are the mean ± S.D. of at least three experiments.

4. Discussion

Oral BH4 supplementation in responsive PKU patients is turning out to be a successful therapy, along with a progressive relaxation or complete withdrawal from dietary therapy [6–9]. A major issue in the understanding of BH4 responsiveness in PKU patients is the knowledge of the disease mechanism once the genetic defect is determined, providing useful information on which to base therapeutic strategies tailored to each patient.

Experimental evidence has accumulated suggesting the molecular basis of the response is multifactorial [10,11,15]. A potential effect of BH4 on PAH gene expression was initially put forward, although results in transgenic mice deficient for cofactor biosynthesis do not support this hypothesis [15]. The data obtained in hepatoma concur with the reported observations. The mRNA analysis rules out transcriptional regulation or mRNA stabilization by BH4, and corroborates that PAH is stabilized by increased BH4 levels, resulting in a net increase in the amount of steady state levels of the enzyme and thus of the activity. It is not uncommon for protein stability to be enhanced by the binding of its cofactor [21–23] and in recombinant PAH, the binding of BH4 was reported to result in reduced proteolysis by trypsin [24].

With the present study, we cannot discard an effect of BH4 on translational regulation as has been described for vitamin B12, cofactor of methionine synthase [25,26]. However, the stabilization of the PAH protein by BH4 alone may account for the increase in steady-state levels of PAH protein observed.

The results obtained in hepatoma cells with the inhibitors of the BH4 biosynthetic pathway are not straightforward. Dicumarol and DAHP have been used previously to investigate cytokine-induced nitric oxide production in endothelial and smooth muscle cells [16,17,27]. However, at 100 μM, dicumarol reduced drastically hepatoma cell viability and mRNA analysis showed a large variability even for housekeeping genes such as GAPDH or 18S rRNA. To circumvent this, we used DAHP which functions as a BH4 mimetic to engage feedback inhibition of GTPCH mediated by the GTPCH feedback regulatory protein (GFRP) [28]. At 10 mM, DAHP results in an unexpected increase in steady-state levels of PAH protein and thus of the relative activity, without affecting mRNA levels or protein turnover. This suggests there could be some unforeseen effect maybe on translation which could account for the results.

Three mutant proteins (A309V, V388M and Y414C) involved in the in vivo response and previously studied in the cell-free in vitro synthesis system TnT [10,11] have been analyzed in hepatoma, which provides a more physiological milieu correlating with the in vivo situation. The three mutant proteins tagged with a FLAG epitope were found to be stabilized by increased BH4 levels, confirming previous results. Wild-type protein did not increase substantially, similar to what was observed in the TNT system [10,11]. Differences in expression levels between endogenous PAH in hepatoma and PAH synthesized in vitro in the TNT system or in transient expression under a viral promoter may account for these results.

The chaperon-like effect of BH4 preventing accelerated degradation is likely not limited to a few PAH proteins, as shown here, but represents a generalised effect on most missense folding PAH mutants explaining the high number of mutations associated to the in vivo response. As it has already been put forward, almost all responsive patients have at least one missense change resulting in a partially active protein and which represent mainly folding defects (excluding a few K_M mutants) [29]. The present studies and those performed in transgenic mice [15,30] firmly support the notion that the stabilization of PAH proteins by BH4 is the major mechanism in vivo responsible for BH4 responsiveness in PKU patients.

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