Effects of dietary glycerol on the expression of pterin carbinolamine dehydratase in the rat

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Earlier studies have shown that the abundance of hepatic phenylalanine hydroxylase (PAH) diminishes to 60 % of control values in rats fed with a diet composed of 40 % (w/w) glycerol [Guerin, Walsh, Donlon and Kaufman (1998) *Int. J. Biochem. Cell Biol.* 30, 1047–1054]. In this experimental model, there are corresponding decreases in the hepatic concentrations of both the hydroxylase cofactor, tetrahydrobiopterin, and the nucleotide guanosine triphosphate. We now show that the cytoplasmic activities of hepatic pterin-4a-carbinolamine dehydratase (PCD) are also lower in these animals, by approx. 50 % compared with control values. Immunoblotting confirmed a diminution of protein abundance *in vivo*. PCD also functions as a dimerization cofactor (DCoH) for the hepatocyte nuclear factor 1 α (HNF1 α)

and the relative abundance of PCD/DCoH in the nucleus is also decreased. There is a small reduction in the mRNA levels for PAH and for PCD/DCoH in the glycerol-fed animals. In the kidney, there is also a diminution in the abundance of both PAH and PCD proteins. Hepatic GTP cyclohydrolase I activity was not altered and the abundance of hepatic HNF1 α remained unchanged. HNF1 α is required for the expression of PAH in the liver and our results support a role for PCD/DCoH, through its interaction with HNF1 α , in regulating the expression of PAH.

Key words: carbinolamine dehydratase, glycerol, hepatocyte nuclear factor 1 α , phenylalanine hydroxylation, regulation, tetrahydrobiopterin.

INTRODUCTION

Phenylalanine hydroxylase (PAH; EC 1.14.16.1) catalyses the first and rate-limiting step in intracellular phenylalanine catabolism in mammals [1]. This enzyme is only expressed in liver and kidney with some 20 % of the total activity being associated with the latter organ [2–4]. The naturally occurring cofactor has been identified as tetrahydrobiopterin (BH₄) [5]. In addition, several synthetic pterins have been prepared, such as dimethyltetrahydropterin (DMPH₄), which has higher cofactor activity [6]. The enzyme is defective in the genetic disease, phenylketonuria [7]. PAH has been studied extensively, as have many of the factors involved in its post-translational control [1]. Earlier studies [8,9] have focused on aspects of its transcriptional control.

After the initial conversion of phenylalanine to tyrosine by PAH, the cofactor intermediate pterin-4a-carbinolamine is formed. Identified originally [10] as a stimulator of PAH, pterin-4a-carbinolamine dehydratase (PCD; EC 4.2.1.96) catalyses [11] the conversion of the carbinolamine to quinonoid dihydrobiopterin, which is reduced subsequently [12] to BH₄ by dihydropteridine reductase (EC 1.6.99.7). After cloning and sequencing, PCD has been found to be identical [13,14] with the dimerization cofactor (DCoH), a cofactor for the hepatocyte nuclear factor 1 α (HNF1 α). HNF1 α -binding sites have been identified in the PAH gene [9] and expression of PAH is dependent on functional HNF1 α [8].

Studies in this laboratory have used animal models, where the activities of PAH can be stimulated during experimental diabetes or attenuated on feeding either glycerol or fructose [15]. We have also monitored changes in the hepatic concentration of biopterin resulting from diabetes or dietary manipulation. In addition, we observed that the concentrations of GTP in freeze-

clamped livers correlated closely with PAH activities and with biopterin concentrations [15]. The present study explored further these correlations and attempted to identify factors involved in the regulation of the expression of PAH and the phenylalanine hydroxylating system *in vivo*. We show here that the relative abundance of PCD/DCoH is also attenuated on feeding glycerol. Our observations provide further insights into the regulation of phenylalanine hydroxylation in the rat and should also provide a model system for studying other genes regulated by HNF1 α .

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (in groups of four) were used throughout the study. A 40 % (w/w) glycerol diet was prepared as described previously [1] and administered for either 5 or 7 days. Control animals were fed with a diet of standard laboratory chow.

Enzyme assays

PAH was assayed using the natural cofactor BH₄ and the synthetic cofactor DMPH₄ as described previously [16]. GTP cyclohydrolase I (GTP-CH) was assayed by quantifying neopterin phosphate using HPLC and fluorescence detection [17]. PCD was assayed by measuring its stimulation of PAH [18,19]. PAH was purified from rat liver as described previously [20]. Nuclei were prepared from rat liver samples by sucrose-gradient centrifugation and nuclear proteins extracted by ammonium sulphate precipitation essentially as described in [21].

Abbreviations used: BH₄, tetrahydrobiopterin; DCoH, dimerization cofactor; DMPH₄, dimethyltetrahydropterin; GTP-CH, GTP cyclohydrolase I; HNF1 α , hepatocyte nuclear factor 1 α ; HRP, horseradish peroxidase; PAH, phenylalanine hydroxylase; PCD, pterin-4a-carbinolamine dehydratase.

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Biopterin determinations

Liver samples were prepared as described previously [15]. Biopterin concentrations were determined by HPLC using fluorescence detection [22].

Western blotting

SDS/PAGE and Western blotting were performed according to standard methods [23]. PCD/DCoH antibody was a gift from the laboratory of Seymour Kaufman (National Institute of Mental Health/National Institutes of Health, Bethesda, MD, U.S.A.). Tissue extracts were loaded on to a 12 % (w/v) acrylamide gel, and after electrophoresis were transferred to PVDF membranes (Amersham Pharmacia). Membranes were blocked in TBST [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.05 % Tween 20] containing 5 % (w/v) non-fat dry milk powder. Rabbit anti-rat PCD/DCoH was added in a 1:10 000 dilution and incubated overnight. The secondary antibody used was horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Sigma), diluted 5000-fold in TBST buffer containing 5 % non-fat dry milk and incubated for 2–3 h. For HNF1 α detection, 10 % (w/v) acrylamide gels and PVDF membranes were used. Rabbit anti-HNF1 α antibody (Geneka Biotechnology; now trading as Activ Motif, Rixensart, Belgium) was added at a 1:1000 dilution and HRP-conjugated secondary antibody was used at a 1:5000 dilution. For PAH detection, 10 % (w/v) SDS gels were used and proteins were transferred to nitrocellulose membranes (Amersham Pharmacia). Rabbit anti-rat PAH from this laboratory was added at a 1:10 000 dilution and HRP-conjugated secondary antibody added at a 1:20 000 dilution. In all cases, blots were washed thoroughly after each incubation step with TBST. Chemiluminescent signals were developed with SuperSignal[®] substrate (Pierce) and membranes exposed to X-ray film (Kodak). Film exposure and development were optimized for each immunoblot. Relative intensity of each band was determined by densitometry (Biorad Fluor-S[™] Multi-imager using Multi-analyst PC software).

Northern-blot analyses

RNA was isolated from hepatic liver samples using RNeasy[®] total RNA isolation system (Promega) according to manufacturer's instructions. The RNA was quantified, subjected to formaldehyde gel electrophoresis, blotted on to nylon membranes (Amersham Pharmacia) and probed with a PCR-generated cDNA fragment according to standard procedures [24]. β -Actin was used in reprobing blots to normalize the RNA samples for densitometry analysis.

Generation of cDNA probes

cDNA was synthesized from total RNA isolated from a control liver sample using Superscript II RNase H⁻ reverse transcriptase (Gibco BRL) and oligo(dT) (Roche). Primers were designed to generate cDNA-specific fragments using the PCR for both PAH and PCD/DCoH. The primers used for PAH were 5'-primer (5'-CGATATCAACCTGACACACA-3') and 3'-primer (5'-CGATCC-ATGCCTAATGTACTG-3'), which yielded a 646 bp product. The primers used for PCD were 5'-primer (5'-ATGGCTGGCAAG-GCACACAG-3') and 3'-primer (5'-CTGGGACACAACTGAG-GCTA-3'), which yielded a 578 bp product. The cDNA probes were radiolabelled with ³²P by random priming for use in the hybridization procedure.

Statistics

Data were analysed statistically using one-way ANOVA and Scheffé's test.

Table 1 Effects of a 40 % glycerol diet on rat hepatic GTP-CH and PCD activities

Values are means $\pm$ S.D. for four animals per group. * Statistical significance (versus control) $P = 0.0004$. Other differences are not significant.

	GTP-CH specific activity (nmol $\cdot$ h ⁻¹ $\cdot$ mg of protein ⁻¹)	Hepatic PCD cytoplasmic specific activity (nmol $\cdot$ min ⁻¹ $\cdot$ mg of protein ⁻¹)
Control	0.057 $\pm$ 0.008	13.37 $\pm$ 2.37
Day 5	0.062 $\pm$ 0.024	3.97 $\pm$ 1.57*
Day 7	0.057 $\pm$ 0.011	5.23 $\pm$ 0.50*

Figure 1 Correlation of PCD activity with PAH activity and BH₄ concentration

PCD and PAH specific activities are expressed as nmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹ and BH₄ concentration in terms of μ g/g. Results are shown as the means $\pm$ S.D. for groups of four animals. Days 5 and 7 refer to length of time for which animals were administered the glycerol-rich diet. Statistical analyses were performed using ANOVA and Scheffé's test. P values (versus controls) ≤ 0.0004 .

RESULTS AND DISCUSSION

GTP-CH activities

GTP-CH (EC 3.5.4.16) catalyses the conversion of GTP to dihydroneopterin triphosphate, the first step in the biosynthesis of BH₄ *de novo*. It is known that this enzyme is regulated by the availability of its substrate GTP [25]. The activities of this enzyme were examined to see if they correlated with those of PAH and with BH₄ levels. The results obtained show that there was no significant change in hepatic GTP-CH activities of the glycerol-fed animals when compared with control values (Table 1). In rats fed with glycerol, the concentrations of hepatic GTP are diminished to approx. 60 % of control values [14]. Thus the finding that there is no change in the abundance of hepatic GTP-CH in those animals is consistent with earlier evidence [25] that the biosynthesis of BH₄ is regulated by the availability of GTP.

Hepatic PCD activities

PCD catalyses the conversion of the carbinolamine, formed from the hydroxylation reaction, to the corresponding quinonoid dihydropterin. The activities of this enzyme were determined in control and glycerol-fed animals and were found to decrease in the latter (Table 1). Statistical analyses showed a significant difference and implied that there is correlation with both PAH activities and BH₄ concentrations *in vivo* (Figure 1). To verify that the alteration

Figure 2 Western-blot analysis of hepatic PCD/DCoH

Analysis was performed as described in the Materials and methods section to determine the relative abundance of the protein *in vivo* on feeding with a glycerol-rich diet. **(A)** Liver cytoplasmic extracts: lanes 1–3, control (40, 20 and 10 μ g total protein respectively; relative A values 1.00, 0.67 and 0.22 respectively); lane 4, glycerol day 5 (40 μ g total protein, A 0.66); lane 5, glycerol day 7 (40 μ g total protein, A 0.55). **(B)** Liver nuclear extracts: lanes 1 and 2, control (60 and 30 μ g total protein respectively; relative A values 1.0 and 0.57 respectively); lane 3, glycerol day 5 (60 μ g total protein, A 0.54); lane 4, glycerol day 7 (60 μ g total protein, A 0.56).

in PCD activity reflected a lower abundance of the dehydratase, Western blotting was performed (Figure 2).

The observation that the levels of PCD decrease over the time course of feeding with 40% glycerol diet is of interest on several counts. First, it demonstrates that the activities of the enzyme correlate both with PAH activities and the levels of hepatic BH₄. Secondly, since PCD also functions as a DCoH of HNF1 α , it suggests that PCD/DCoH may also be responsible for controlling hepatic PAH activities, and indeed plays an integral part in the regulation of the hydroxylation cycle as a whole through its interaction with HNF1 α . Potential binding sites for the transcription factor are present in the rat hydroxylase gene [26], and the requirement of functional HNF1 α in the expression of PAH has been documented [8]. However, relatively little is known about the regulation of PCD. Inactivation of HNF1 α by homologous recombination in mouse leads only to a partial reduction in the expression of PCD/DCoH [8]. It has also been observed that the enzymic activity of PCD/DCoH is not essential for binding to HNF1 α [27].

Hepatic nuclear PCD/DCoH

In view of the role of PCD as a DCoH for the transcription factor HNF1 α , it was of interest to determine if there was any differential effect on nuclear PCD activities in the glycerol-fed animals and the controls. PCD was purified originally from liver cytoplasmic extract as the PAH-stimulating protein [18]. It has been reported that during purification of the enzyme, the pellet fraction of liver homogenate did not contain any detectable PCD activity [28]. However, PCD/DCoH was isolated from a rat nuclear extract [13] and found to co-purify with HNF1 α . Therefore nuclear extracts were prepared from control and glycerol-fed animals and assayed for PCD activity. No significant activity was detected in these samples. It was reported previously that the amount of hepatic nuclear PCD/DCoH is approx. 2% of that of cytoplasmic PCD/DCoH [29]. To address the question of whether or not there is a change in the levels of PCD/DCoH, nuclear extracts were also analysed by Western blotting. It was found that the reduction in the nuclear abundance of PCD/DCoH in the glycerol-fed animals (Figure 2B) was similar to that in cytoplasm.

These results show that the distribution ratio of PCD/DCoH between the cytoplasm and the nucleus remains the same in both

Figure 3 The effects of glycerol-rich diet on PAH mRNA abundance *in vivo*

Isolation of total RNA and Northern-blot analysis were performed as described in the Materials and methods section. **(A)** Membrane hybridized with PAH probe; **(B)** membrane hybridized with β -actin probe; and **(C)** rRNA detected by staining with ethidium bromide. Total RNA loaded on to each lane was 20 μ g. Lane 1, control, relative A of 1.0; lane 2, glycerol day 5, A 0.68; lane 3, glycerol day 7, A 0.81; values given are from optical densitometry of PAH mRNA relative to β -actin mRNA transcripts.

the control rats and those on the high glycerol diet, even though the total PCD/DCoH levels are decreased in the latter. The decrease in PCD/DCoH, available for DCoH function, suggests that HNF1 α cannot function as strongly in its role as a transcription factor for the set of hepatic genes that it regulates in the glycerol-fed rats. Since PAH is one of these genes, this suggests that PCD/DCoH, by its interaction with HNF1 α , also regulates the expression of rat hepatic PAH in addition to its role in regenerating BH₄.

Northern-blot analyses

Experimental results from enzyme assays and Western-blot analysis have shown that the protein levels of both PAH and PCD/DCoH are reduced in animals fed with a 40% glycerol diet. The next issue to be addressed was whether or not these changes also occur at the transcriptional level. In most cases, changes in the levels of a particular mRNA transcript are reflected in the amount of protein that is translated. Thus because of the significant decrease seen in both PAH [15] and PCD/DCoH proteins, their respective messenger RNAs were quantified. Northern-blot experiments were therefore performed to examine if the decrease in enzyme levels was just at the protein level or due to a decrease in the level of mRNA transcripts.

The results showed that there was a decrease in the levels of both PAH and PCD/DCoH mRNAs in animals fed with a 40% glycerol diet when compared with control values (Figures 3 and 4). However, these reductions were of a lower degree when compared with those of protein levels. Reduction in mRNA levels for both PAH and PCD/DCoH was not as obvious as the reduction at the protein level as expected. Regarding PCD/DCoH it is evident that post-transcriptional regulation also occurs. It has been reported that in the expression of human PCD/DCoH the synthesis of its mRNA and its protein do not seem to proceed in a parallel manner. The amount of PCD/DCoH mRNA in human liver is only four times that in human epidermal keratinocytes and dermal fibroblasts. However, the protein level in the liver is 20-fold higher than in the latter two cell types [29]. Thus, in the liver, it appears that a minor change in mRNA levels results in a more significant effect on the amount of PCD/DCoH protein synthesized.

Figure 4 Effects of glycerol-rich diet on PCD/DCoH mRNA abundance *in vivo*

Membrane was hybridized with (A) PCD/DCoH probe and (B) with β -actin probe; (C) rRNA detected by staining with ethidium bromide. Total RNA loaded on to each lane was 20 μ g. Lane 1, control, relative A of 1.0; lane 2, glycerol day 5, A 0.78; lane 3, glycerol day 7, A 0.71; values given are from optical densitometry of PCD/DCoH mRNA relative to β -actin mRNA transcripts.

Figure 5 Western-blot analysis of HNF1 α in hepatic nuclear extracts

Lanes 1 and 2, control (30 and 60 μ g total protein respectively; relative A values 0.6 and 1.0 respectively); lane 3, glycerol day 5 (60 μ g total protein, A 1.0); lane 4, glycerol day 7 (60 μ g total protein, A 1.1).

Hepatic HNF1 α

Because of its pivotal role in regulating PAH gene expression, and in the light of the DCoH function of the dehydratase, the relative abundance of HNF1 α in hepatic nuclear extracts was determined using Western-blot analysis. It was found that hepatic HNF1 α protein levels remain unchanged in the glycerol-fed animals when compared with controls (Figure 5). Since PAH mRNA transcripts have been already shown to be decreased in animals fed with a high glycerol diet, this suggests that it is the reduced levels of PCD/DCoH protein that mediate a reduced transactivation of the PAH gene, whereas the abundance of HNF1 α remains unperturbed.

Effects of high glycerol diet on renal PAH and PCD activities

Since both the activity of PCD and its relative abundance are decreased in the liver of the glycerol-fed animal, we decided to examine the activities and abundance of PCD in kidney and to check for possible correlation with PAH. Renal PAH activities (most notably DMPH $_4$ -dependent activities) were decreased in animals fed with a high glycerol diet for 7 days (Table 2). The decrease is due to lower amounts of the enzyme present as verified by immunotitration (G. Walsh and J. Donlon, unpublished work).

As for liver tissue samples, PCD activity in the kidney was determined using an indirect assay that measured the stimulation of purified PAH after the addition of kidney extract. The results (Table 2) showed that no significant change occurred in renal PCD activities in the glycerol-fed animals, which is in contrast with liver where a significant decrease in activity occurred (Table 1).

Table 2 Effects of a 40% glycerol diet on rat kidney PCD and PAH activities

Values are means $\pm$ S.D. for four animals in each group. * Statistical significance (versus control) $P = 0.016$. Other differences not significant.

	Renal PCD cytoplasmic specific activity (nmol $\cdot$ min $^{-1}$ $\cdot$ mg of protein $^{-1}$)	Renal DMPH $_4$ -dependent PAH specific activity (nmol $\cdot$ min $^{-1}$ $\cdot$ mg of protein $^{-1}$)
Control	4.37 $\pm$ 0.47	2.30 $\pm$ 0.24
Day 5	4.40 $\pm$ 0.33	1.61 $\pm$ 0.37*
Day 7	4.83 $\pm$ 0.57	1.77 $\pm$ 0.23*

Figure 6 Western-blot analysis of kidney tissue extracts

(A) PCD/DCoH in cytoplasmic extracts: lanes 1–3, control (60, 45 and 30 μ g total protein respectively; relative A values 1.0, 0.76 and 0.38 respectively); lane 4, glycerol day 5 (60 μ g, A 0.70); lane 5, glycerol day 7 (60 μ g, A 0.65). (B) PAH in cytoplasmic extracts: lane 1, purified liver PAH standard; lanes 2–4, control (12.5, 25 and 50 μ g total protein respectively; A values 0.55, 0.86 and 1.00 respectively); lane 5, glycerol day 5 sample (50 μ g total protein, A 0.82); lane 6, glycerol day 7 sample (50 μ g total protein, A 0.70).

Western-blot analysis, however, showed that there was a decrease in the amount of renal PCD in the glycerol-fed animals when compared with controls (Figure 6). This decrease was of a lower order than that seen in the liver. That a decrease can be seen with immunoblotting can be explained by the chemiluminescent detection system being more sensitive than the dehydratase-dependent hydroxylase stimulation assay.

When hepatic and renal PAH activities were compared, it could be seen that the renal enzyme was in a much more activated state as indicated by the BH $_4$ /DMPH $_4$ activity ratio. Also, kidney PAH cannot be further activated by any of the mechanisms that stimulate the BH $_4$ -dependent activity of the rat liver enzyme *in vitro* [1]. This suggests that the renal enzyme is not post-translationally regulated in the same fashion as hepatic PAH. Our current findings also identify a further difference with respect to the regulation of PAH expression in these two tissues. In HNF1 α -deficient animals, only hepatic PAH expression is affected [30]. HNF1 α is predominant in the liver, whereas in the kidney, HNF1 α and HNF1 β are present in roughly equal amounts [30]. The normal renal expression of PAH is unaffected, probably because HNF1 β takes over the role of HNF1 α for this function in the kidney. This illustrates that differential regulation of renal PAH occurs at the transcriptional as well as the translational level.

Conclusion

The present study, along with earlier observations [15], shows that correlation exists between components of the phenylalanine hydroxylating system. Expression of PAH is regulated tightly, and the transcription factor HNF1 α is integral for its expression. The role of DCoH in stabilizing HNF1 α dimers and increasing transcriptional activation has been established and we now have shown that diminished PCD/DCoH protein levels *in vivo* are

correlated with reduced abundance of both PAH enzyme and BH₄. Whereas bipterins do not appear to play a role in the formation of DCoH/HNF1 α tetramers [31] this does not exclude the possibility of a transcriptional co-activation role for BH₄.

The mechanism of PCD/DCoH regulation remains undefined. Reduced transcription of the gene in the HNF1 α knock-out mouse [8] suggests that HNF1 α has a role in controlling PCD/DCoH expression, and through its dimerization function PCD/DCoH may play a role in its own regulation. Enzyme function is not required for the dimerization activity [27] and the co-localization of PCD/DCoH with HNF1 α in cells where hydroxylase activity is absent demonstrates the distinct and separate functions of the protein. Very recent evidence [32] from studies with DCoH knock-out mice shows decreased PAH activity and a corresponding reduction in mRNA abundance. Interestingly, a homologue of DCoH, termed DCoH2, which also has dimerization activity, was identified. The physiological model used here has been valuable in exploring the role of PCD/DCoH in the regulation of expression of PAH. Further studies are required to clarify the precise control point in the phenylalanine hydroxylating system, and for the determination of how PCD/DCoH itself is regulated, not only within the hydroxylating system but also in cells devoid of hydroxylation activity. The differential control of expression of the phenylalanine hydroxylating system in the liver and kidney has yet to be understood fully.

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