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ORIGINAL ARTICLE

Erythrocyte and Liver Porphobilinogen Deaminase in Cirrhosis and Clinical or Experimental Cholestasis

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ABSTRACT

BACKGROUND Porphobilinogen deaminase, the third enzyme in the haem synthetic process -mainly expressed in the erythrocytes and liver - has a key role in the pathogenesis of the acute porphyrias.

DESIGN AND RESULTS We studied the effect of cirrhosis or cholestasis on this enzyme activity and found that:

1. Erythrocyte porphobilinogen deaminase was significantly increased (p=0.0003) in 27 patients with non-alcoholic liver cirrhosis (19.89±6.65 μmoles/h.l) and 24 patients with extrahepatic cholestasis (20.69±11.17 μmoles/h.l) as compared to 30 controls (12.77±4.76 μmoles/h.l). Its activity was positively correlated to the prothrombin time in both patient groups and negatively to the alkaline phosphatase in the cholestasis group.

2. Erythrocyte porphobilinogen deaminase of controls significantly increased when their plasma was substituted by that of patients with cholestasis (p<0.001) or cirrhosis (p=0.05), although it remained lower than that of the latter. No change was observed in samples from patients with cirrhosis or cholestasis when their plasma was substituted by that of controls.

3. In 8 rabbits, cholestasis produced by ligation of the common bile duct significantly increased porphobilinogen deaminase both in the erythrocytes (from 30.26±10.33 to 48.87±15.82 nmoles/h.l, p=0.002) and the liver (from 13.27±4.79 to 17.68±5.42 nmoles/h.g, p=0.035). In 8 sham-operated rabbits erythrocyte porphobilinogen deaminase also increased (from 29.60±9.85 to 33.32±12.23 nmoles/h.l, p=0.016), but to a significantly lower degree than that of the "cholestatic" group (111.10±10.95 % versus 167.96±41.64% p=0.006) while the hepatic enzyme remained unchanged (from 13.40±3.85 to 13.83±7.21 nmoles/h.g, p=0.80).

4. In 5 patients with cholestasis the mean hepatic PBG-D activity was higher than in the 5 controls (12.63±3.24 versus 9.64±1.17 nmoles/h.g), although not significantly higher (p=0.11).

CONCLUSION PBG-D activity is considerably increased in liver cirrhosis and clinical or experimental cholestasis. It seems probable that plasma factors may play a role in this effect by inducing PBG-D activity.

INTRODUCTION

Haem is synthesized through a complex pathway involving eight enzymes [1]. Decreased activity of any of these enzymes -except for the first one, δ-aminolevulinic acid synthetase (ALA-S) - results in the accumulation of some by-products of the metabolic procedure and, thus, in the pathogenesis of the porphyrias [2]. These uncommon metabolic disorders are classified either as acute and non-acute, according to the presence of neurologic symptoms, or as erythropoietic and hepatic, according to the tissue where the metabolic defect predominates. In most cases, ALA-S is secondarily induced, thus further increasing the accumulation of by-products.

Porphobilinogen deaminase (PBG-D) is the third enzyme of the haem synthetic pathway and the second in increasing order of activity after ALA-S. It is genetically deficient in acute intermittent porphyria and relatively insufficient in most other acute porphyrias [1]. Several drugs and chemicals are known to affect ALA-S activity and this knowledge is of important clinical significance. On the contrary, little is known about factors affecting PBG-D activity and it seems to be unresponsive to any intervention. Some decades ago, Blum et al [3] reported that erythrocyte PBG-D activity was increased in a group of patients with chronic hepatic diseases, mainly of alcoholic etiology. The aim of the present study was to further investigate this finding.

METHODS

The whole study was performed in four phases:

FIRST PHASE

Eighty-one subjects - all with a good nutritional status, a mean daily alcohol consumption during the last year no more than 10 g, and normal creatinine values were included in this phase. They were classified in three groups (I-III) as follows:

Group I (controls): Thirty healthy subjects (16 male/14 female, mean age 54.3 yr) who were not taking any medication.

Group II (*cirrhotic)*: Twenty-seven patients with non-alcoholic liver cirrhosis (17 male/10 female, mean age: 61.7 yr). Four of them had also developed hepatocellular cancer. A coexisting malignancy was excluded in the remaining 23 patients by a negative liver ultrasonography (in most cases also by a computed tomography) and a normal α-fetoprotein value. Seventeen patients (13 male, 4 female) were HBsAg-positive (3 of them were also HCV-positive), 7 patients (4 male, 3 female) were only HCV-positive, 2 patients (both female) had autoimmune hepatitis and in 1 female patient the etiology of cirrhosis was unknown.

Group III (cholestatic): Twenty-four patients with extrahepatic bile duct obstruction (14 male/10 female, mean age: 57.2 yr) due to a bile stone in 18 and to pancreatic cancer in 6. In some of them, vitamin K was parenterally administered. They all had no anaemia.

In all studied subjects, erythrocyte PBG-D activity was measured in whole blood (haemolysed with the addition of a 0.2% Triton-X-100 solution) according to the method by With and Pedersen [4]. The results are expressed as micromoles of uroporphyrinogen synthesised per liter of red blood cells in 1 hour. In the patients of groups II and III, serum total bilirubin, alanine aminotransferase, alkaline phosphatase, albumins, total cholesterol and prothrombin time -expressed as international normalised ratio (INR)- were also measured. In most of the studied subjects a reticulocyte count was also measured.

SECOND PHASE

It was performed in 15 subjects -five from each group- randomly selected among those included in the first phase. Eight ml of blood were taken from one subject of each group and the plasma was separated by a refrigerated centrifuge. The erythrocytes were twice washed with an isotonic KCl solution and then blood samples were "restored" by mixing 1 ml of plasma with 1 ml of erythrocytes in the following combinations:

- "normal" plasma/"normal" erythrocytes (No/No)
- "cirrhotic" plasma/"cirrhotic" erythrocytes (Ci/Ci)
- "cholestatic" plasma/"cholestatic" erythrocytes (Ch/Ch)
- "normal" plasma/"cirrhotic" erythrocytes (No/Ci)
- "normal" plasma/"cholestatic" erythrocytes (No/Ch)
- "cirrhotic" plasma/"normal" erythrocytes (Ci/No)
- "cholestatic" plasma/"normal" erythrocytes (Ch/No).

All samples were immediately haemolysed (with the addition of a 0.2 % Triton-X-100 solution) and remained for 30 minutes in room temperature. Then erythrocyte PBG-D activity was measured as previously. Results were expressed as percentage of the No/No value.

THIRD PHASE

Sixteen male white rabbits were randomly divided in two equal groups (A, B). In the morning of the operation day, blood was taken from an ear vein to measure serum bilirubin (total, conjugated), alkaline phosphatase and erythrocyte PBG-D activity. After that the rabbits were operated as follows, using morphine and ketamine for anaesthesia:

Group A: In these animals after laparotomy the common bile duct was ligated and a liver specimen weighing approximately 0.5 gr was excised.

Group B: In these animals laparotomy and liver biopsy were also performed but without ligation of the common bile duct.

Hepatic PBG-D activity was measured in the liver specimens as we have previously described [5]. The results were

expressed as nanomoles of uroporphyrinogen synthesised per 1 gr of liver tissue in 1 hour. Three days after the operation the animals were sacrificed. Blood samples and liver specimens were taken to repeat the same measurements.

FOURTH PHASE

Ten patients who were operated in the hepatobiliary region were included in this phase after giving their informed consent. Five of them underwent cholecystectomy for gallstones without cholestasis (non-cholestatic group). The other 5 had obstructive jaundice due to a common bile duct stone (cholestatic group). Since barbiturates -and possibly other drugs- may affect PBG-D activity [5], anaesthesia was obtained in all 10 patients by the use of the same drugs (thiopental, midazolam, isoflurane, fentanyl and vecuronium). PBG-D activity was measured in a liver specimen excised during the operation.

STATISTICAL ANALYSIS

One-tailed analysis of variance, including the Bonferroni t-test, Student's t-test for paired and unpaired observations as well as univariate and multivariate regression analysis were used for the statistical evaluation of our results.

RESULTS

FIRST PHASE (table I, figure 1)

Patients with either cirrhosis ($p<0.005$) or cholestasis ($p<0.01$) had a significantly higher PBG-D activity than the controls ($F=8.91$, $p=0.0003$). There was not a significant difference of the enzymic activity between cirrhotic patients with or without hepatocellular cancer ($p= 0.90$) or between patients with cholestasis due to a bile stone or to pancreatic cancer ($p=0.75$). The reticulocyte count was 1.78 ± 0.63 % in 17 of the controls, 1.40 ± 0.38 % in 24 of the cirrhotic patients and 1.77 ± 0.88 % in 18 patients with cholestasis. These values did not differ significantly among the three groups ($F= 2.23$, $p= 0.12$).

In the cirrhotic patients, univariate analysis showed that PBG-D values were significantly correlated positively to those of INR ($r=0.8641$, $p<0.001$) and negatively to those of serum albumins ($r=-0.8125$, $p<0.001$). Multiple regression analysis showed ($R=0.8972$, $F=13.76$, $p<0.0001$) this correlation to be significant only with INR ($p=0.0037$). In the cholestatic patients univariate analysis showed a significant negative correlation of PBG-D to alkaline phosphatase ($r=-0.9196$, $p<0.001$) while multiple regression analysis revealed ($R=0.9520$, $F=27.38$, $p<0.0001$) a significant correlation negatively to the alkaline phosphatase ($p<0.0001$) and positively to the INR ($p=0.031$).

SECOND PHASE (table II)

The substitution of their homologous plasma with that of normal subjects did not actually change erythrocyte PBG-D

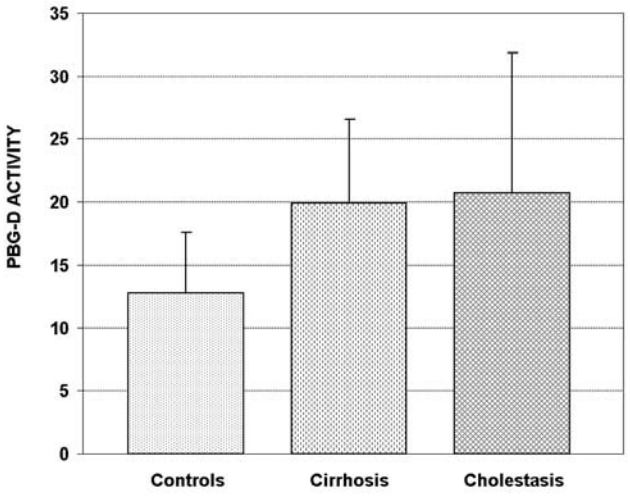

FIGURE 1. Erythrocyte porphobilinogen deaminase activity (mean, standard deviation) in controls and patients with cirrhosis or cholestasis.

TABLE I. Erythrocyte PBG-D activity in controls and patients (in μmoles/h.l)

	number	mean ± SD
Controls	30	12.77 ± 4.76
Cirrhosis (total)	27	19.89 ± 6.65
Cirrhosis (no cancer)	23	19.50 ± 1.92
Cirrhosis (plus cancer)	4	19.96 ± 7.20
Cholestasis (total)	24	20.69 ± 11.17
Cholestasis (bile stone)	18	20.25 ± 11.40
Cholestasis (cancer)	6	22.00 ± 11.38

TABLE II. PBG-D activity in the mixed samples of the second phase (expressed as percentage of the No/No* value)

	mean ± SD
Ci/Ci*	146.80 ± 22.99
Ch/Ch*	148.60 ± 13.70
No/Ci*	143.80 ± 21.22
No/Ch*	145.40 ± 16.82
Ci/No*	117.20 ± 14.17
Ch/No*	124.60 ± 5.03

* their meaning is explained in the text

activity in samples from patients with cirrhosis (t=0.21, p=0.84) or cholestasis (t=0.33, p=0.75). On the contrary, PBG-D activity of normal subjects significantly increased when their plasma was substituted by that of patients with either cirrhosis (t=2.71, p=0.05) or cholestasis (t=10.94, p<0.001). However, even increased, the enzymic activity remained significantly lower than that of the respective blood samples from patients with cirrhosis (Ci/Ci versus Ci/No: t=2.45, p=0.04) or cholestasis (Ch/Ch versus Ch/No: t=3.68, p=0.014).

THIRD PHASE (table III, figure 2)

In group A, PBG-D activity significantly increased after bile duct ligation both in the erythrocytes (t=4.87, p=0.002) and the liver (t=2.62, p=0.035). There was also a considerable increase in total and conjugated bilirubin and alkaline phosphatase. The simple laparotomy of group B caused a smaller but also significant increase in the erythrocytic (t=3.18, p=0.016) but not in the hepatic PBG-D (t=0.26, p=0.80). Bilirubin and alkaline phosphatase values remained unchanged. The percent increase of erythrocyte PBG-D in group A was significantly higher than in group B (167.96±41.64 % versus 111.10±10.95%, t=3.74, p=0.006). No significant correlation of the PBG-D activity increase to that of bilirubin or alkaline phosphatase was found.

FOURTH PHASE

The mean hepatic PBG-D activity in patients with cholestasis was higher than in those of the non-cholestatic group (12.63±3.24 versus 9.64±1.17 nmoles/h.g). However, the difference was not statistically significant (t=1.94, p=0.11).

DISCUSSION

Although PBG-D has a key role in most acute porphyrias [1], little is known about factors that may affect its activity. In order to further investigate the aforementioned observation by Blum et al. [3] -later confirmed by Kaczynski et al. [6] - we performed the present study. We initially measured erythrocyte PBG-D activity in non-alcoholic patients since alcohol is known to affect per se haem synthesis [7]. We found it significantly increased both in the patients with cirrhosis (mean increase 55.76%) and in those with extrahepatic cholestasis (mean increase 62.02%) as compared to normal controls. We did not exclude from the study cirrhotic patients with coexistence of hepatocellular cancer since a previous study [6] showed that it does not affect PBG-D activity. This was confirmed in our study too. The etiology of cholestasis (benign or malignant cause of obstruction) did not seem to affect the increase in enzymic activity.

In the cirrhotic group we found a significant positive correlation of PBG-D to the INR and a possible negative one to the serum albumins. Since malnourished patients were excluded from the study both, INR and serum albumins level

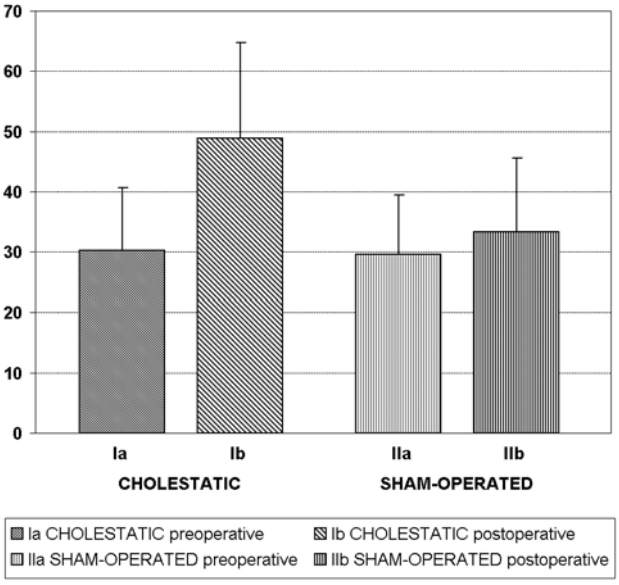

FIGURE 2. Erythrocyte porphobilinogen deaminase activity (mean, standard deviation) pre-and post-operatively in cholestatic and sham-operated rabbits.

TABLE III. Pre- and post-operative values in cholestatic and sham-operated rabbits (mean ± standard deviation)

	bilirubin total (mg/dl)	bilirubin conjugated (mg/dl)	con-alkaline phosphatase (IU)	erythrocyte PBG-D (nmoles/h.l)	liver PBG-D (nmoles/h.l)
CHOLESTATIC					
preoperative	0.24±0.12	0.13±0.05	178.25±32.82	30.26±10.33	13.27±4.79
postoperative	6.54±4.07	4.61±3.06	378.38±216.25	48.87±15.82	17.68±5.42
SHAM-OPERATED					
preoperative	0.33±0.12	0.15±0.09	180.38±41.83	29.60±9.85	13.40±3.85
postoperative	0.31±0.11	0.12±0.06	167.38±35.23	33.32±12.23	13.83±7.21

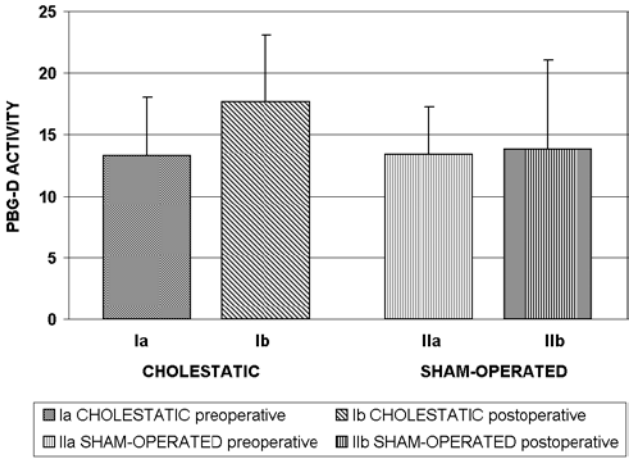

FIGURE 3. Liver porphobilinogen deaminase activity (mean, standard deviation) pre-and post-operatively in cholestatic and sham-operated rabbits.

can be accepted as reliable indices of the severity of hepatic failure. So it may be suggested that by-products, not sufficiently metabolised by the liver as normally, or factors involved in the pathogenesis of liver injury are responsible for the increase in the enzymic activity. In the second case, one would expect a good correlation of PBG-D activity to the alanine aminotransferase, which, however, was not found in our study.

In the cholestatic group PBG-D activity was also increased. However, it was negatively correlated to the alkaline phosphatase, an index of the severity of cholestasis. Thus, patients with mild cholestasis had higher PBG-D values than those with a severe one. A possible explanation for this paradox is that the increase in PBG-D activity is indirectly produced through changes in liver function due to the cholestasis, but if cholestasis is severe enough it can reverse this effect possibly by a direct inhibitory action on the same enzyme. In favour of that possibility is the positive correlation of PBG-D to the INR, also observed in this group, although we must mention that in patients with cholestasis, INR may be increased not only due to affected liver function but also due to malabsorption of vitamin K, because of the cholestasis. Some of our patients, however, were already receiving vitamin K parenterally, which could reverse the effect of cholestasis on INR.

Could the effect of cholestasis be attributed to retention of substances as bilirubin or bile acids? Kohasi et al [8] found, that, in rats, bilirubin -a molecule with considerable similarity to hydroxymethylbilane, the product of the reaction catalysed by PBG-D - was a reversible, non-competitive inhibitor of purified rat liver PBG-D whereas several bile acids had no effect on it. With regard to humans, only indirect information is offered by a study in patients with Gilbert's syndrome according to which PBG-D activity was normal although bilirubin was high enough and did not change when bilirubin returned to normal levels [9]. In our study, although all patients were jaundiced, bilirubin values were not significantly correlated to those of PBG-D. In a few experiments (unpublished data), we added chemically pure bilirubin to the hemolysate from normal blood samples and 30 minutes later we measured PBG-D activity. The aimed concentration of 10 mg/dl of blood could not be achieved because of the low water solubility of bilirubin. Nevertheless, the enzymic activity was moderately decreased.

The increase of PBG-D activity in the patients with cirrhosis or cholestasis may be due either to increased synthesis or to direct induction of the enzyme. The findings of the second phase of our study (cross-incubations) indicate that some factor(s) present in the serum of patients with cirrhosis or cholestasis can directly increase PBG-D activity of normal erythrocytes and not induce its synthesis since mature erythrocytes do not contain DNA. The observed increase was lower than that in the erythrocytes of the respective patients. This finding may be due either to the fact that this effect is time dependant and could not be achieved completely in the relatively short preincubation period (30 minutes) or to the fact that there is also an increased production of PBG-D in these patients. The erythrocytes from patients with either cirrhosis or cholestasis kept their increased PBG-D activity when their homologous plasma was substituted by that of controls. This finding indicates that the "inducing effect" of these conditions, if achieved, is maintained even in the absence of plasma factors during the incubation period.

A crucial question for us was whether liver PBG-D activity is also increased since acute porphyrias are all mainly of hepatic origin. We found that cholestasis produced in rabbits by bile duct ligation caused an increase of PBG-D whose mean value was higher than that of the sham-operated animals by 56.86% in the erythrocytes and 43.60% in the liver. A similar increase (31.02%) in liver PBG-D was found in our patients (fourth phase) although the difference from the controls was not statistically significant, possibly because of the small number of cases. So it seems that in both patients and experimental animals, cholestasis increases PBG-D activity and this effect is possibly more pronounced in the erythrocytic than in the hepatic enzyme. It must be noted that Piper et al. [10] have also noted an increase in PBG-D activity by nearly 40% in rats with experimental cholestasis. The reason why the enzymic activity was - to a significantly lesser degree- also elevated in the red cells of sham-operated animals is unclear. One may speculate that it was due to the operative stress or to the drugs used for anaesthesia -although we did not use drugs known to affect haem synthesis.

In conclusion, PBG-D activity is considerably increased in cholestasis and cirrhosis and plasma factors seem to play a role in it. Future studies may reveal these factors and possibly offer a novel approach to the management of patients with acute porphyria.

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