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Vitamin D-binding protein as a biomarker of active disease in acute intermittent porphyria☆

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ABSTRACT

Acute intermittent porphyria (AIP) is an autosomal dominant metabolic disorder caused by a deficiency of hepatic porphobilinogen deaminase (PBGD). The disease is characterized by life threatening acute neurovisceral attacks. The aim of this study was to identify metabolites secreted by the hepatocytes that reflect differential metabolic status in the liver and that may predict response to the acute attack treatment. Plasma vitamin D binding protein (VDBP) from a mouse model of AIP displayed an abnormal migration in 2D-electrophoresis that is efficiently recovered upon gene therapy leading to liver specific over-expression of the PBGD protein. The change in VDBP mobility results from a differential isoelectric point suggesting a post-translational modification that takes place preferably in the liver. Liquid chromatography–mass spectrometry (LC–MS) analysis of human samples before and after glycosidase treatment revealed glycosylated plasma VDBP specifically in patients with recurrent attacks of AIP. Glycosylated VDBP recovered normal values in three severely afflicted AIP patients submitted to therapeutic liver transplantation. Our findings suggest that post-translational modification of VDBP might be considered as a promising biomarker to study and monitor the liver metabolic status in patients with AIP.

Significance: We describe an increased glycosylation of VDBP in porphyric livers. Normal glycosylation was recovered upon liver gene therapy in a mouse model of porphyria or after liver transplantation in severely afflicted patients with AIP. Moreover, quantification of glycosylated VDBP by our ELISA immunoassay or LC–MS protocol in patients undergoing *PBGD*-gene therapy (www.aipgene.org) may be used as a marker indicating improvement or normalization of the patient's hepatic metabolism.

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1. Introduction

Acute intermittent porphyria (AIP, MIM 176000) is an autosomal dominant metabolic disease caused by a deficiency of hepatic porphobilinogen deaminase (EC 4.3.1.8; PBGD), the third enzyme of the heme synthesis pathway [1,2]. Although most carriers of PBGD mutations (>80%) do not develop symptoms, some carriers may become symptomatic presenting with sporadically acute neurovisceral attacks while a few may have repeated attacks of varying severity (chronic active disease) [3]. The onset of symptoms almost never occurs before puberty, and women in fertile age are more afflicted than men, implying special susceptibility to reproductive hormones [3]. Acute attacks are caused by exposure to certain endogenous or exogenous factors that up-regulate the hepatic heme synthesis pathway. However, it is not unusual that precipitating factor cannot be identified.

Under conditions of increased heme demand of hepatic heme synthesis, the activity of the first and rate limiting enzyme in heme synthesis pathway, aminolevulinate synthase (ALAS1) is greatly increased. Consequently, the precursors, aminolevulinic acid (ALA) and porphobilinogen (PBG), the metabolites prior to the deficient enzymatic step PBGD, accumulate. The concentration of PBG in urine is invariably increased in individuals with symptomatic AIP and also in a number of asymptomatic/latent AIP carriers. During an acute attack, urinary PBG concentrations are often more than 10–20 times the upper reference limit. PBG concentrations decrease during remission but may remain increased for months or years [1,4,5].

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The aim of this work was to identify plasma biomarkers that may differentiate between degrees of metabolic engagement in the porphyric liver and predict the response to different modalities of AIP treatment irrespective of the heme precursor accumulation. The liver is a highly metabolically active organ that secretes numerous metabolites into the blood. Thus, the hypothesis of this work was that intermittent or chronic up-regulation of the hepatic heme synthesis pathway or decreased heme availability may induce hepatic metabolism dysfunction and consequent changes in the plasma profile of liver secreted proteins.

Circulating vitamin D binding protein (VDBP), also known as group-specific component globulin or VTDB (UniProtKB/Swiss-Prot entry P02774, Proteomic databases Q6LDC6 — http://www.uniprot.org) is a monomeric glycoprotein predominantly produced by hepatic parenchymal cells [6]. It circulates at a high concentration in the serum (6–7 μM), being part of the albumin gene family and sharing the multiple disulfide linked triple domain structure of albumin [7]. VDBP plays pivotal roles in multiple biological and metabolic pathways, such as the organ-specific transport of vitamin D and its metabolites to target organs [8]. VDBP binds both the inactive and the active form of vitamin D obtained after enzymatic hydroxylation in the liver and kidney [9]. However, only 5% of total VDBP binding sites have been found to be occupied by vitamin D, which suggests a function beyond vitamin D transport [10,11]. VDBP has also been shown to play crucial roles *in vivo* as an extracellular actin-scavenger system [12]. It promotes macrophage activation [13] and differentiation [14,15], binds fatty acids [16], stimulates osteoclasts [17], enhances the chemotactic activity of C5-derived peptides, and associates with immune cell surfaces such as T and B cells [10].

There is no known natural deficiency of VDBP in any vertebrate species but a DBP null mouse has been generated. This mouse is healthy [18] without phenotypical features of vitamin deficiency [19,20]. In humans, plasma VDBP concentrations have been found to be unaffected by age, weight, Body Mass Index or obesity [21]. Various studies have demonstrated the relevance of plasma VDBP levels as a marker in different clinical conditions. VDBP plasma concentration is decreased shortly after trauma, largely because of its increased consumption within the actin-scavenging system [22–24]. Low circulating levels of VDBP are also described in fulminant hepatic failure [25], advanced liver fibrosis [26], cystic fibrosis [27] and in conditions with hyperinsulinemia and insulin resistance in humans [28]. Previous reports suggest that VDBP products possess two independent O-glycosylation sites occupied by linear $(NeuNAc)_1(Gal)_1(GalNAc)_1$ trisaccharide at T420 and a $(Gal)_1(GalNAc)_1$ disaccharide at T418, but remain free of N-glycosylation [29,30].

A mouse model of AIP exhibits the typical biochemical characteristics of human porphyria in the absence of liver injury or necrosis [31]. Plasma VDBP obtained from these AIP mice displayed an abnormal migration when run in 2D-electrophoresis. Moreover, ELISA immunoassay revealed a reduction of the circulating plasma VDBP obtained from the AIP mice as compared to wild type animals. Of interest, targeted hepatic *PBGD*-gene therapy for AIP induced normalization of both levels and migration patterns of plasma VDBP, suggesting altered post-transcriptional modification that takes place in the liver of the porphyric mice, i.e., before the protein is secreted to the blood. In this study, we developed an accurate mass spectrometry method able to determine and monitor plasma VDBP levels in patients with AIP. We have confirmed that plasma VDBP secreted by parenchymal hepatic cells exhibited a different glycosylation pattern in patients with signs of active disease.

2. Material and methods

2.1. Studies in a murine model of AIP

AIP mice are compound heterozygotes of two different disruptions of the pbgd gene [31]: T1 strain (C57BL/6-pbgd$^{tm1(neo)Uam}$) and T2 strain (C57BL/6-pbgd$^{tm2(neo)Uam}$). AIP mice exhibit a hepatic PBGD activity reduced to 33% of normal and a porphyrin precursor excretion within the normal range [32]. In these mice, a massive increased excretion of heme precursors in urine was induced after treatment with porphyrinogenic drugs such as phenobarbital. In order to rule out the effect of the heme-precursor over-excretion, none of the animals used in this study received phenobarbital or any other drug. We also analyzed an additional group of AIP animals treated with recombinant Adeno-Associated Virus (AAV)-mediated liver *PBGD*-gene therapy [32]. Recombinant human *PBGD* transgene is under the control of the liver-specific human α-1-antitrypsin promoter with regulatory sequences from the human albumin enhancer (EalbAAT promoter). The recombinant serotype 5 particles were produced and purified as previously described [32]. Experimental protocols were approved by the Ethics Committee of the University of Navarra (CEEA022-06) according to European Council Guidelines.

2.2. Study design and study population

A retrospective, cross-sectional analysis of VDBP levels used plasma samples from 52 healthy adult individuals (26 men and 26 women) and 27 women carrying a mutation in the *PBGD* gene (6 presenting with recurrent acute attacks and 21 without symptoms at the time of sample collection). We also analyzed VDBP in plasma samples from three AIP patients, two women [33] and one man (unpublished case), with a severe clinical picture of recurrent active chronic porphyria acute attacks during many years obtained before and after combined liver and kidney transplantation (CLKT) [33,34]. Details of the two female patients are reported in both articles; ref [33] described CLKT and ref [34] is more a description of the metabolic situation during end-stage renal failure. Two patients with active porphyria cutanea *tarda* were also included. Porphyria cutanea *tarda* is the most prevalent subtype of porphyrias and is characterized by a high accumulation of plasma porphyrins of hepatic origin. Blood samples were collected in tubes containing EDTA or heparin as an anticoagulant. Samples were centrifuged for 15 min at 1,000 ×*g* within 30 min of collection, aliquoted and then stored at −20 °C to avoid repeated freeze–thaw cycles. Given that chronic kidney disease is a long term complication in patients with chronic active AIP [35], renal impairment at the time of sample collection was estimated by plasma cystatin C concentration (ref: RD191009100, BioVendor GmbH, Heidelberg, Germany). All AIP patients were informed of the aims of the study and agreed to participate by signing an informed consent form. The research was conducted in accordance with the Declaration of Helsinki Principles.

2.3. Plasma VDBP measurements by ELISA immunoassay

An ELISA sandwich protocol was developed to quantify plasma VDBP from both humans and mice. The VDBP sequence of human (Supplementary Fig. 1) and mouse (NM_008096 XM-132170) origin showed a 78% identity (370aa/474aa) and a similarity of 89.7% (425aa/474aa). The anti-mouse antibody VDBP (ref: AF4188, R&D Systems, Abingdon, UK) that recognized both the human and murine protein was chosen as a capture antibody to coat the bottom of the 96-well plates. Capture antibody was diluted 1/250 in phosphate buffered saline (PBS 1×) at pH 7.4; 100 μl/well of the coating solution was incubated overnight at 4 °C. Then, the coated wells were washed thrice with PBS-0.05% Tween and the remaining protein-binding sites were blocked by adding 200 μl assay diluent (ref: 555213, BD Pharmingen, San Agustin de Guadalix, Spain). Plates were covered with an adhesive plastic and incubated for at least 1–2 h at room temperature. Plates were washed thrice with PBS-0.05% Tween and 100 μl of appropriately diluted samples was added to each well (sample dilution in assay diluent for mouse was 1/20 and for human 1/4,000). Standard curve (duplicates) and blanks were run with each plate to ensure accuracy. Plates was incubated for 90 min at room temperature with gentle shaking and, then