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Comprehensive analysis of the tryptophan metabolome in urine of patients with acute intermittent porphyria

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ABSTRACT

Background: Acute intermittent porphyria (AIP) is a rare metabolic disorder due to a deficiency of porphobilinogen deaminase, the third enzyme of the heme biosynthetic pathway. This low enzymatic activity may predispose to the appearance of acute neurological attacks. Seminal studies suggested that AIP was associated with changes in tryptophan homeostasis with inconclusive results. Therefore, the aim of this study was to analyze the urinary metabolome of AIP patients focusing on tryptophan metabolism using state-of-the-art technology.
Methods: This was a case-control study including a group of 25 AIP patients with active biochemical disease and increased excretion of heme-precursors and 25 healthy controls. Tryptophan and related compounds and metabolites including: large neutral amino acids (LNAAs), serotonin, kynurenine, kynurenic acid and anthranilic acid were quantified in urine by liquid chromatography tandem-mass spectrometry (LC–MS/MS). Twenty-nine biological markers (including metabolic ratios and absolute concentrations) were compared between patients and controls.
Results: Significant differences were found in the tryptophan-kynurenine metabolic pathway. Compared to controls, AIP patients showed: (a) increased urinary excretion of kynurenine and anthranilic acid ($P < 0.005$); (b): elevation of the kynurenine/tryptophan ratio ($P < 0.001$) and (c): decrease of the kynurenic acid/kynurenine ratio ($P = 0.001$). In contrast, no differences were found in the serotonin metabolic pathway independently of the markers and ratios used.
Conclusions: The results of the study demonstrate that there is an imbalance in the kynurenine metabolic pathway in AIP patients, with an increase of the kynurenine/tryptophan ratio in urine and a reduction of the kynurenic acid/kynurenine ratio. The modified ratios suggest induction of indoleamine 2,3-deoxygenase and decreased activity of kynurenine aminotransferase in the liver. The results confirm that LC–MS/MS is useful for the characterization of the urinary metabolome of hepatic porphyrias.

1. Introduction

Acute intermittent porphyria (AIP) is a rare disease which is due to a deficiency of porphobilinogen deaminase (PBGD, EC 2.5.1.61), the third enzyme of the heme biosynthetic pathway [1]. Carriers of mutations within the *PBGD* gene are at risk of presenting acute neurovisceral attacks associated with the overproduction of heme precursors δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) [2].

Abbreviation: AIP, acute intermittent porphyria; LNAAs, large neutral amino acids; LC–MS/MS, liquid chromatography tandem-mass spectrometry; PBGD, porphobilinogen deaminase; ALA, δ-aminolevulinic acid; PBG, porphobilinogen; ALAS-1, 5-aminolevulinate synthase; Trp, tryptophan; TPH, tryptophan hydroxylase; MAO, monoamine oxidase; IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; KAT, kynurenine aminotransferase; KYNU, L-Kynurenine hydrolase; KMO, kynurenine 3-monooxygenase; LHRH, luteinizing hormone-releasing hormone; Val, valine; Leu, leucine; Ile, isoleucine; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; 5-HT, serotonin; 5HIAA, 5-hydroxyindoleacetic acid; Kyn, kynurenine; KA, kynurenic acid; 3OHKyn, 3-hydroxykynurenine; XA, xanthurenic acid; AA, anthranilic acid; OHAA, 3-hydroxyanthranilic acid; SsC, spearman's correlations

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Acute attacks manifest as a dysfunction of the central, peripheral and autonomous nervous systems. A wide range of psychiatric manifestations are frequently presented [3–5]. Acute attacks occur due to hyperactivity of 5-aminolevulinate synthase (ALAS-1), the first enzyme of the heme biosynthetic pathway that catalyzes the formation of ALA from succinyl-CoA and glycine. ALAS-1 hyperactivity combined with partial PBGD deficiency may induce sudden accumulation of heme-precursors. Hepatic overproduction of exportation of neurotoxic ALA is the most plausible explanation for the acute crisis, however, the etiopathogenesis of most neurological manifestations of AIP remains unknown. Intravenous injection of hemin, which temporarily restores hepatic heme and inhibits ALAS-1 is the most effective treatment to reverse acute clinical symptoms [3–5]. New therapeutic approaches based on silencing overexpression of the *ALAS-1* gene are currently under evaluation [6].

In the majority of AIP patients, clinical remission is not associated with a rapid decline of heme-precursors overproduction since the urinary levels of PBG/ALA remain elevated for years [7]. In some patients, the sustained deregulation of the heme-synthesis pathway is associated with recurrent neurological crises, while others may remain free of acute symptoms even while maintaining high PBG/ALA excretion. The sustained hepatic deregulation of heme-synthesis in AIP may be associated with major biochemical abnormalities. However, since AIP is a hepatic disease, the assessment of disturbances occurring in the liver is problematic if only a limited number of metabolites in blood or urine are estimated. In contrast, a comprehensive metabolomic analysis of a large number of analytes may be a useful tool.

In a previous study, we used liquid chromatography-tandem mass spectrometry (LC–MS/MS) to investigate the urinary steroid metabolome in AIP by simultaneously measuring 55 steroid hormones and metabolites [8]. Here, we present a follow-up study covering the metabolism of tryptophan (Trp) and related intermediates.

In a seminal study published in 1961, J M Price reported that patients with acute forms of porphyria exhibited abnormal metabolism after oral administration of L-Trp [9]. Subsequent studies in rodents and humans observed modifications but failed to provide conclusive results [10,11]. Moreover, it was hypothesized that enhanced plasma levels and brain uptake of Trp, as an indirect consequence of hepatic heme deficiency, was associated with acute attacks [12,13].

The aim of this study was to analyze the urinary metabolome of AIP patients with active disease focusing on tryptophan metabolism by comprehensive target analysis of 29 markers using LC–MS/MS in AIP patients compared to healthy controls.

2. Materials and methods

2.1. Patients

We studied 25 adult Caucasian Spanish patients with biochemically active AIP (23 women and 2 men, ranging in age from 22 to 54 years). All these patients had initially presented an acute porphyria attack, had been diagnosed with AIP and were regularly attended in the Porphyria Unit of the Hospital Clinic of Barcelona for clinical follow-up. AIP was assessed by biochemical and enzymatic analyses according to European Porphyria Initiative recommendations and external quality assessment schemes [14]. Genetic analysis of the *HMBS* synthase gene confirmed AIP in all cases.

The patients presented a variable clinical condition with some remaining fully asymptomatic while others presented intermittent acute episodes. However, all patients had been acute symptom-free for a minimum of two months at the time of urine collection for this study. On the other hand, chronic complaints such as altered mood states, depression, fatigue or pain in the back were frequently reported. Three patients presenting frequent recurrent attacks were on a prophylactic heme-arginate regime (Normosang@; 3 mg/Kg; every 2–3 weeks). In these latter patients, the urine was collected before the heme-arginate infusions. None of the patients included in the study were receiving luteinizing hormone-releasing hormone (LHRH) agonists.

Independently of the clinical status, all the patients presented increased long-term urinary excretion of the heme precursors PBG and ALA.

None of the patients presented other diseases in addition to AIP. Liver function assessed by classical serum biomarkers was normal. Renal function was strictly normal in all the cases included, with two AIP patients with renal disease being excluded from the study.

All AIP patients reported compliance with the dietetic and life-style recommendations aimed at minimizing the risk of new acute attacks.

Twenty-five healthy volunteers (23 women and 2 men; age 25–45 years) were recruited from the laboratory staff and included in the study as controls. They all presented normal renal and liver function.

All the patients were informed of the purpose of the study and provided signed consent for the collection of urine samples for a specific metabolomic study related (only) to their porphyria disease. The confidentiality of the databases and the results was explained to the participants by Drs Aguilera and To-Figueras (staff of Hospital Clínic of Barcelona). The project was assessed and approved by the Ethics Committee for Clinical Investigation (CEIC; "Comité Ético de Investigación Clínica") of the Hospital Clínic of Barcelona (HCB)-Fundació Clínic per Recerca Biomèdica (FCRB). Second morning urine from all patients and controls was obtained between 0900 h–1000 h in carefully controlled conditions on the hospital premises. Aliquots were immediately protected from light and frozen at −80 °C until analyses.

2.2. PBG and ALA measurements

Ion-exchange chromatography using the ALA/PBG column test (Bio-Rad GmbH, Munich, Germany) was used to quantify PBG and ALA. The analysis of creatinine and liver enzymes was done by standard methods using ADVIA 2400 equipment (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA). PBG and ALA concentrations were normalized to creatinine (mmol/mol of creatinine).

2.3. Standards and chemicals

Reference standards of valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), serotonin (5-HT), 5-hydroxyindoleacetic acid (5HIAA), kynurenine (Kyn), kynurenic acid (KA), 3-hydroxykynurenine (OHKyn), xanthurenic acid (XA), anthranilic acid (AA) and 3-hydroxyanthranilic acid (OHAA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standards were obtained from Toronto Research Chemicals (Toronto, Canada) and Alsachim (Illkirch-Graffenstaden, France). For detailed information see reference [15].

Formic acid (LC/MS grade), acetonitrile and methanol (LC gradient grade) were supplied by Merck (Darmstadt, Germany). Ammonium formate (HPLC grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was provided using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.4. Quantification of urinary markers

Urinary markers were quantified using a method reported previously [15].

The LC–MS/MS system used to perform the analysis was an Acquity UPLC system (Waters Associates, Milford, MA, USA) coupled to a Quattro Premier triple quadrupole mass spectrometer (Waters Associates) equipped with an electrospray ionization interface. The chromatographic separation was carried out in an Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm) (Waters Associates) at a flow rate of 300 μL min^{-1}. Water and methanol both with ammonium formate (1 mM) and formic acid (0.01% v/v) were used as mobile phase solvents. A gradient elution was used for the chromatographic