DOI: 10.1111/eci.12102

ORIGINAL ARTICLE

Circadian rhythms in acute intermittent porphyria – a pilot study

Sebastian Larion[*], Frederick R. Caballes[*,†], Sun-Il Hwang[*,‡], Jin-Gyun Lee[*,‡], Whitney E. Rossman[*,‡], Judy Parsons[‡], Nury Steuerwald[*,‡], Ting Li[*,‡], Vinaya Maddukuri[*,†], Gale Groseclose[*,‡], Carla V. Finkielstein[§] and Herbert L. Bonkovsky[*,‡]

[*]The Liver-Biliary-Pancreatic Center, Carolinas HealthCare System, Charlotte, NC, USA, [†]Department of Medicine, Carolinas HealthCare System, Charlotte, NC, USA, [‡]Department of Research, Carolinas HealthCare System, Charlotte, NC, USA, [§]The Integrated Cellular Responses Laboratory, Department of Biological Sciences, Virginia Tech University, Blacksburg, VA, USA

ABSTRACT

Background Acute intermittent porphyria (AIP) is an inherited disorder of haem synthesis wherein a partial deficiency of porphobilinogen (PBG) deaminase (PBGD) with other factors may give rise to biochemical and clinical manifestations of disease. The biochemical hallmarks of active AIP are relative hepatic haem deficiency and uncontrolled up-regulation of hepatic 5-aminolevulinic acid (ALA) synthase-1 (ALAS1) with over-production of ALA and PBG. The treatment of choice is intravenous haem, which restores the deficient regulatory haem pool of the liver and represses ALAS1. Recently, haem has been shown to influence circadian rhythms by controlling their negative feedback loops. We evaluated whether subjects with AIP exhibited an altered circadian profile.

Materials and methods Over a 21-h period, we measured levels of serum cortisol, melatonin, ALA, PBG and mRNA levels (in peripheral blood mononuclear cells) of selected clock-controlled genes and genes involved in haem synthesis in 10 Caucasian (European-American) women who were either postmenopausal or had been receiving female hormone therapy, six of whom have AIP and four do not and are considered controls.

Results Four AIP subjects with biochemical activity exhibited higher levels of PBG and lower levels and dampened oscillation of serum cortisol, and a trend for lower levels of serum melatonin, than controls or AIP subjects without biochemical activity. Levels of clock-controlled gene mRNAs showed significant increases over baseline in all subjects at 5 a.m. and 11 p.m., whereas mRNA levels of ALAS1, ALAS2 and PBGD were increased only at 11 p.m. in subjects with active AIP.

Conclusions This pilot study provides evidence for disturbances of circadian markers in women with active AIP that may trigger or sustain some common clinical features of AIP.

Keywords 5-aminolevulinic acid, acute intermittent porphyria, circadian rhythms, clock-controlled genes, cortisol, melatonin.

Eur J Clin Invest 2013; 43 (7): 727–739

Introduction

Acute intermittent porphyria (AIP) is an inborn error of haem synthesis caused by autosomal dominant mutations in PBGD, the third enzyme of the haem biosynthetic pathway, which carries out the polymerization of the monopyrrole porphobilinogen (PBG) into hydroxymethylbilane (HMB) [1–6]. These defects, acting in concert with other genetic, environmental and nutritional factors, may give rise to relative hepatic haem deficiency and uncontrolled up-regulation of hepatic ALA synthase-1, normally the rate-controlling enzyme for hepatic haem synthesis. Such induction leads to over-production and over-excretion of ALA and PBG in the urine, both of which are hallmarks of active AIP. Elevated levels of ALA, or a non-PBG product derived therefrom, are believed to be responsible for many of the symptoms common in biochemically active AIP [1–7]. Elevations of urinary and/or plasma levels of ALA and PBG are key criteria clinicians use to make a diagnosis of an acute porphyria. Along with resolution of symptoms, decreases in levels of ALA and PBG are helpful for gauging efficacy of therapy of acute porphyric attacks. Concentrations of these precursors to haem in plasma and urine have been shown to normalize quickly, albeit transiently, following intravenous haem therapy. They rapidly and permanently fall to normal

following successful liver transplantation accompanied by complete and permanent remission of clinical presentation, suggesting an hepatic origin of the disease [8–10]. Other evidence for the key role of ALA, as against PBG, in causing symptoms comes from observations of similar clinical features (severe bouts of abdominal or other pain, etc.) in subjects with acute lead poisoning or hereditary tyrosinemia, type I, in which marked inhibition of ALA dehydratase (also known as PBG synthase), the second enzyme of the haem synthetic pathway, gives rise to marked over-production and over-excretion of ALA without increased PBG [11,12]. Similar biochemical changes also are seen in the rare form of acute porphyria due to severe homozygous or compound heterozygous deficiency of ALA dehydratase [1–6].

The clinical features of AIP typically include diffuse gastrointestinal pain, vomiting and constipation, as well as tachycardia and hypertension [1–6]. Severe attacks can include development of peripheral neuropathies, including pain, muscle weakness and CNS features such as delirium, mental confusion and seizures. Although the exact pathogenesis of acute porphyric attacks remains unclear, it is well known that women are more likely to develop porphyric attacks than men, especially during the luteal phase of their menstrual cycles [13]. Moreover, gonadotropin-releasing hormone analogues have been shown to reduce or eliminate cyclical attacks in some women [14]. These observations, along with other complaints such as disorders of mood and sleep (insomnia), have led to speculation that AIP attacks may be related to hormonal imbalances [1,2,4–6]. Anterior pituitary hormones are secreted as pulses in a rhythmic manner, and these irregularities in mood and sleep may partly be caused by alterations in gonadal hormones. As postmenopausal women have decreased gonadal hormones, fluctuations in sex hormones are but one of several possible reasons responsible for the altered mood states and sleep disorders. Another possible factor influencing irregularities in mood and sleep is alteration in the circadian release of melatonin [15,16].

Disruption of circadian rhythms, the mechanisms that adjust our physiology to external environmental signals, has emerged as a new potential risk factor in the development of numerous diseases and disorders. These rhythms are deranged by shift work and jet lag [17], and in disparate conditions such as insomnia, sleep syndromes [18], acute myocardial infarction and depression; such disruption is an important factor that contributes to cancer development and progression [19,20]. Circadian oscillations respond to three components: an input signal (e.g. light and temperature) that entrains the body rhythms to environmental cues, the suprachiasmatic nucleus that integrates signals and a third component known as the output pathway that results in, for example, hormone secretion or a locomotor activity. The levels of cortisol and melatonin in serum are accepted as standards of intrinsic clock activity [21,22].

Cellular haem levels are controlled in part by circadian expression of the haem-degrading enzyme, haem oxygenase-1, activity of which peaks at night [23,24]. Haem, in turn, controls the activities of a variety of signal transducers and transcriptional regulators, including the circadian transcription factors Npas2, Rev-erb alpha and Per2, which are responsible for driving the positive and negative feedback loop of the intracellular clock and which accumulate at night [25–27]. The Npas2 factor forms heterodimers with its counterpart Bmal1 to regulate the expression of target genes; thus, cellular haem status controls the binding of Npas2 to DNA [28]. Kaasik and Lee showed that haem-Npas2 regulates *ALAS1* expression and that haem also differentially modulates the expression of *Per1* and *2* genes [24]. Moreover, Per2, a transcription factor that accumulates at night, enhances Npas2/Bmal1 activity towards the *Alas1* gene, whereas partial ablation of both *Per* genes in mice results in circadian disruption of *ALAS1* and *2* expression [29]. Binding of haem occurs in two distinct regions of hPER2, including a novel haem regulatory motif located near the carboxyl terminus of the protein. Interestingly, proteasome-mediated degradation of hPER2 is exclusively associated with haem-mediated oxidation and ligand binding to the carboxyl-terminus domain. In agreement, haem synthesis directly controls hPER2 levels *in vivo*, and its specific binding to the haem regulatory motif influences the period length and phase-shifting properties of the clock in synchronized cells [27]. The current model suggests the existence of reciprocal regulatory loops between haem biosynthesis and circadian protein expression. Thus, dysregulation of any of these pathways will impact cell homoeostasis and could be a determining factor for disease initiation and progression.

These observations prompted us to hypothesize that subjects with AIP, especially those with biochemical activity, indicative of relative hepatic haem deficiency, may demonstrate abnormalities in normal circadian rhythms and that these may help to account for some of the common complaints of subjects with AIP, such as difficulties with sleep and alterations of mood and affect. We present here results of a pilot study comparing the circadian rhythmicity of serum cortisol and melatonin, haem precursor concentrations and selected clock-controlled gene expression in control and AIP subjects.

Methods

Chemicals

5-aminolevulinic acid (ALA) (MW: 167·59) and PBG (MW: 244·24) were purchased from Frontier Scientific (Logan, UT). Heavy isotope-labelled versions, namely 5-amino-2,2-levulinic-2,2-d2 acid HCl (MW: 169·59) and carbon-13 labelled

porphobilinogen (MW: 246·24), were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada) and Frontier Scientific (Logan, UT), respectively. A porphyrin standard mixture (porphyrin acids chromatographic marker) was purchased from Frontier Scientific (Logan, UT). Creatinine was purchased from Sigma-Aldrich (St. Louis, MO). Oasis HLB solid phase extraction cartridge (1 cc, 30 mg), used in sample clean-up, was purchased from Waters Corp. (Milford, MA). Water, acetonitrile and formic acid (FA) of mass spectrometry grade were purchased from EMD Chemicals, Inc. (Darmstadt, Germany). Unless stated otherwise, all chemicals were of HPLC or analytical grade.

Study subjects

Postmenopausal Caucasian subjects, six with AIP and four sex-matched controls, were enrolled in this study. Postmenopausal women were intentionally selected to avoid the effect of variations in gonadotropic and sex hormones during the menstrual cycle in the circadian release of cortisol and melatonin, thus simplifying data collection and analysis. Additionally, subject C1 did not report having a period for more than 1 year and was thus considered perimenopausal. Due to poor peripheral venous access, one control subject had blood obtained at only the first two time points (11 a.m. and 2 p.m.). All AIP subjects were asymptomatic in regard to typical symptoms of acute porphyric attacks, were postmenopausal and had a well-documented, stable clinical history of AIP with confirmatory genetic testing (Table 1), not needing hospitalization or therapy for AIP in the past 3 months, and did not have other major chronic diseases of any organ or system. On the day of study, two of the subjects with AIP were judged to have no detectable biochemical activity, defined as normal urinary (< 4 uM) and plasma (< 1 uM) levels of PBG, whereas four, although without symptoms, had biochemical activity with mild to moderate increases in urinary and plasma PBG (Table 1, Fig. 3). Control subjects were women without documented AIP or any other forms of porphyria and documented single-void or 24-h urinary ALA and PBG levels within the normal range. All subjects resided in the adjacent eastern or central time zones of the USA and had not travelled to other time zones within 2 months of the study days.

Exclusion criteria for control and AIP subjects were acute psychiatric illness, including but not limited to major depression and seasonal affective disorders (SAD) [30] within 6 months of the study, as well as those requiring psychoactive medications within this period. Those with a history of psychiatric disease in first-degree family members were also excluded. Subjects were screened using the self-report Beck Depression Inventory II (BDI-II), and subjects with scores greater than 19 and thus suggesting moderate depression were excluded. Other exclusion criteria were advanced chronic disease other than porphyria, those currently using oral contraceptive pills currently or within the past 6 months, blood haemoglobin less than 10 g/dL, blood transfusion within 6 months of study, those regularly taking nonsteroidal anti-inflammatory (NSAIDs), beta-blockers [31] or alpha-blockers, acute illness in the past 2 months, travel to another time zone within the preceding 2 months, history of alcohol abuse, treatment with Panhematin® within 3 months of study and inability or unwillingness to give informed consent.

Study protocol and sample collection

All human studies were carried out in a sleep centre in Charlotte, NC, in accordance with the principles of the Declaration of Helsinki and adhered to a protocol approved by the Institutional Review Board of Carolinas Medical Center. Subjects who volunteered for the study were instructed to eat balanced meals and abstain from alcohol for 1 week before the day of the study. Subjects were asked not to exercise vigorously during the study day but were free to move about the sleep centre throughout the day as they would normally. Subjects underwent 15 mL blood draws beginning at 11 a.m. and every 3 h thereafter (1400, 1700, 2000, 2300, 0200, 0500 and 0800), using indwelling IV catheters located in a suitable peripheral vein for the duration of the study period. A solution of heparin in 0·154 M NaCl was used to keep the catheter patent, and approximately 20 mL of blood was removed prior to each sample and replaced into the subjects, followed by approximately 10 mL additional heparinized saline. Samples collected between the hours of midnight and 8 a.m. were collected and processed under dim light of < 30 lux [32,33], using a F8T5 fluorescent red 12″ lamp (Antares Enterprises, Inc., Santa Fe, CA) that has been previously shown to minimally affect the human circadian clock [34]. All sample tubes were wrapped in aluminium foil to prevent any light influence on hormonal or mRNA profiles. Subjects were asked to sleep with eye masks and ear plugs in the dark during the time of their usual bed rest, but were instructed to get out of bed if they felt they had been awake for more than 15 min prior to their expected time of waking. The ambient temperature of the bed room was kept at 23 ± 2 °C via a thermostat.

Whole blood samples were apportioned between serum separator tubes and tubes containing EDTA, an anticoagulant. Blood in serum separator tubes was allowed to clot and then centrifuged, after which the sera were carefully removed and stored in dark brown cryovials stored at −70 °C until further analysis. The EDTA-containing tubes were centrifuged at 3300 *g* for 10 min at room temperature, after which the plasma was removed, aliquoted and stored as above. The buffy coats from the EDTA-containing tube were also collected and flash frozen at −70 °C. At baseline (approximately 11 a.m.), random urine samples were collected in brown containers, kept on ice until they were aliquoted and stored frozen (−70 °C).

Table 1 Selected demographic, clinical and laboratory features of subjects studied

					Baseline urine											
	Subject	Age (year)	BMI (kg/m^2)	History of acute attacks	ALA (μM/mM creatinine)	PBG (μM/mM creatinine)	Total porphyrins (nM/mM creatinine)	Beck depression score	PBGD activity in RBCs (nmol porphyrin/L/s)	Mutational analysis of PBGD gene	Other medical conditions	Sleeping problems	Avg. h of sleep/ night	Coffee intake (cups/d)	Cigarette smoking (ppd)	Alcohol use
Control subjects	C1	33	29·2	No	3·2	1·5	107	1	22	Control	None	No	10·5	0	1	None
	C2	45	25·6	No	2·6	1·6	144	1	21	Control	None	No	5	2·5	1/2	None
	C3	60	26·5	No	1·8	1·0	69	0	22	Control	None	No	7	1	None	None
	C4	48	24·1	No	3·0	1·2	91	0	19	Control	None	No	7	0	None	None
AIP subjects	A1*	68	24·2	No	1·8	1·2	226	5	18	R173W	HTN, high cholesterol, recurrent UTI, restless legs syndrome	Yes	4·5	3	1	None
	A2*	51	22·8	Yes, 8 year ago	3·9	3·4	175	14	17	Splice-site mutation, IVS71 g > a in HMBS allele	No	Yes	6	0	None	None
	A3	47	28·3	No	4·2	5·7	331	2	20	R173W	No	Yes	6	2	1	None
	A4	48	37·6	Yes, 1 year ago	5·0	5·2	417	13	16	R173W	Depression, chronic pain, GERD	Yes	10	2	1	None
	A5	49	33·7	Yes, 1 year ago	4·6	5·1	279	18	19	Deletion in HMBS allele 730-731 delct	Bipolar disorder type II	Yes	5	0	None	None
	A6	78	25·4	Yes, 1 year ago	18	24·3	870	10	10	R167Q	No	Yes	4	2	None	None

a, adenine; A, subject with AIP; AIP, acute intermittent porphyria; ALA, 5-aminolevulinic acid; Avg, average; BMI, body mass index; C, control subject; ct, cytidine–thymidine; d, day; g, guanosine; GERD, gastro-oesophageal reflux disease; HTN, systemic arterial hypertension; IVS, intervening sequence (intron); h, hours; PBG, porphobilinogen; PBGD, PBG deaminase [also known as hydroxymethylbilane synthase (HMBS)]; ppd, pack per day; Q, glutamine; R, arginine; RBCs, red blood cells; UTI, urinary tract infection; W, tryptophan.
*Denotes AIP subjects without evidence of biochemical activity (normal urinary excretions of ALA, PBG, porphyrins and normal levels of ALA and PBG in plasma).

Determination of serum melatonin and cortisol

As intrinsic markers of SCN activity, serum melatonin and cortisol levels were determined using Melatonin ELISA kit (IBL International, Toronto, Ontario, Canada) and Cortisol ELISA kit (ALPCO Immunoassays, Salem, NH), respectively, as per the manufacturer's instructions. Samples were run in duplicate for each analyte and time point. Results were extrapolated using a four-parameter logistics fit calibration curve constructed with five standard concentrations for cortisol (0·5–30 μg/dL) and four standard concentrations for melatonin (7–250 pg/mL). Total hormone levels for the entire 21-h study period were determined by area under the curve (AUC) analysis using the trapezoid method. In this method, hormone levels for 3-h intervals between blood draws were averaged and multiplied by the length of time that passed (i.e. 3 h), and the values were summated to obtain the entire serum hormone concentration over the 21-h study period for each subject.

Expression of clock-controlled and haem metabolism genes

RNA was isolated from peripheral blood mononuclear cells (PBMCs) from buffy coat samples using miRNeasy Mini Kit (Qiagen, Valencia, CA) in accord with manufacturer's instructions. First-strand complementary DNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). The reverse transcription reaction was incubated at 42 °C for 30 min and stopped by heating to 85 °C for 5 min. Fifty nanograms of final product was used as template for PCR. qRT-PCR was performed using Taqman® Probe-Based Detection X with an ABI Prism 7500 Fast Real-Time PCR System using Taqman® gene expression assays and Taqman® Gene Expression master mix (Applied Biosystems, Foster City, CA). Templates were amplified by 40 cycles of denaturation at 95 °C for 15 s, annealing of primers and probe together with extension at 60 °C for one min in triplicate reactions. Fluorescence data were acquired during the combined anneal/extension step. Suitable negative control reactions were run to confirm the absence of DNA contamination. Samples were run in triplicate, and fold change values were calculated using comparative Ct analysis and normalized to an invariant control [TBP (TATA-box-binding protein)].

Analyses of urinary and plasma porphyrins

Urinary porphyrins were initially purified using a solid phase extraction process and subsequently analysed by reversed-phase ultra-high-performance liquid chromatography/fluorescence detection (UPLC/FLD), as previously described [35]. Briefly, 1·0 mL urine samples were purified by solid phase extraction using 1 mL (30 mg) Oasis HLB cartridges prior to injection. (Baseline urine samples were available for this analysis from only 6 of the 10 total subjects enrolled; samples from the other four subjects had been exhausted.) Porphyrins were then separated and quantified by reversed-phase UPLC/FLD system (Shimadzu Nexera/Prominence Hybrid UPLC system, Kyoto, Japan) equipped with a Thermo Hypersil-Gold C_{18} (5 μm, 4·6 × 250 mm; Thermo Scientific, Waltham, MA) column. The mobile phase consisted of 50 mM aqueous ammonium formate (pH 3·0, A) and acetonitrile (B), with a flow rate of 1·5 mL/min and a linear gradient profile as follows: 0 min, A 80%, B 20% to 30 min, A 20%, B 80%. The final 20/80 mixture was run for an additional 5 min (total run time/sample = 35 min). Porphyrins were detected and quantified by fluorescence with a red-sensitive detector (Shimadzu Prominence RF-20A XS, Tokyo, Japan) at excitation wavelength of 398 nm and emission wavelength of 600 nm. Four porphyrins showed good linearity at a concentration range of 25–250 pmol/mL, namely 8-carboxyl porphyrin (uroporphyrin), 7-carboxyl porphyrin, 6-carboxyl porphyrin and 5-carboxyl porphyrin, while two porphyrins showed good linearity at a concentration range of 50–500 pmol/mL, namely 4-carboxyl porphyrin-I (coproporphyrin-I) and 4-carboxyl porphyrin-III (coproporphyrin-III). Porphyrin concentrations were normalized to urinary creatinine as determined by adding heavy isotope-labelled creatinine (+3 Da) for selected reaction monitoring quantification using high-performance liquid chromatography/mass spectrometry (LC–MS; ion transitions: creatinine 114 $m/z \rightarrow 44$ m/z, isotope-labelled creatinine 117 $m/z \rightarrow 47$ m/z). For plasma samples, 500 μL portions were initially diluted with the same volume of water and followed by SPE with the same protocol as above.

Analyses of plasma and urine ALA and PBG

To quantify ALA and PBG in plasma, we used heavy isotope internal standards processed as described above, separated by ultra-high-performance liquid chromatography (UPLC), and quantified by tandem mass spectrometry (MS), as previously described [36–38]. Briefly, 1·0 mL portions of plasma samples were purified using 1 cc (30 mg) Oasis MCX cartridges prior to injection into the liquid chromatography system. The LC–MS system consisted of a Waters UPLC (Milford, MA) equipped with a Phenomenex Kinetex C_{18} (1·7 μm, 2·1 × 150 mm) column and a Thermo-Fisher TSQ Quantum Ultra mass spectrometer, triple quadrupole MS with electrospray ionization (Kyoto, Japan). The spray voltage and capillary temperature were optimized at 3500 V and 272 °C, respectively. The optimal collision energy for ALA and PBG was 25% and 15%, respectively. Heavy isotope-labelled ALA (+2 Da) and PBG (+2 Da) were added as internal standards for relative quantification of endogenous ALA and PBG, which were eluted over a linear gradient mobile phase consisting of 0·1% (v/v) FA in water and 0·1% FA in acetonitrile. The quantification of ALA and PBG

was achieved using single reaction monitoring of parent ions to product ions (ion transitions: ALA 132 *m/z* → 86 *m/z*, isotope-labelled ALA 134 *m/z* → 88 *m/z*; PBG 210 *m/z* → 122 *m/z*, isotope-labelled PBG 212 *m/z* → 124 *m/z*). Total integrated plasma ALA or PBG concentrations during the entire 21-h study period were determined by AUC analysis using the trapezoid method as explained above.

Statistical analyses

A two-way analysis of variance (ANOVA) with a Student Newman–Keuls (SNK) *post hoc* test was used to identify differences in hormone concentrations or mRNA levels and times of day among subject groups. A one-way ANOVA with a SNK *post hoc* test was used to evaluate differences in porphyrin concentrations among subject groups. A one-way ANOVA with a SNK *post hoc* test or Student's *t*-test was used to determine differences in total hormone concentrations in AUC analysis among subject groups. Periodograms of hormone levels or ALA/PBG concentrations plotted against time were created using SigmaPlot 12·3 (Systat Software, Inc., San Jose, CA). Circadian rhythmicity for hormone levels or ALA/PBG concentrations were evaluated using the single cosinor analysis as previously described [39,40], using Cosinor Periodogram 2·3, a statistical software program originally developed by R. Refinetti [Univ South Carolina, Columbia, SC (www.circadian.org/softwar.html)] [41]. Cosinor analysis is a statistical method of determining a least squares line of best fit for a cosine function as described by Nelson *et al.* [42]. Descriptive variables provided by cosinor analysis include period length, rhythm-adjusted mean (MESOR), amplitude and cosine maximum (acrophase). All data are presented as means ± SE, and a value of $P < 0{\cdot}05$ was considered statistically significant.

Results

Summary description of subjects studied

Three control subjects and six subjects with AIP completed the full blood draw schedule. Selected demographic, clinical and laboratory features are summarized in Table 1. Of note is that all AIP subjects reported having persistent sleeping problems, whereas none of the control subjects reported similar difficulties.

Time course and levels of serum cortisol

Cortisol levels were highest in the early morning period in both control and AIP subjects, with significantly more serum cortisol at 8 a.m. than at all or almost all other time points within the respective study groups (Fig. 1a). Mean values of serum cortisol were not different between control and all AIP subjects at any time point (two-way ANOVA). However, average serum

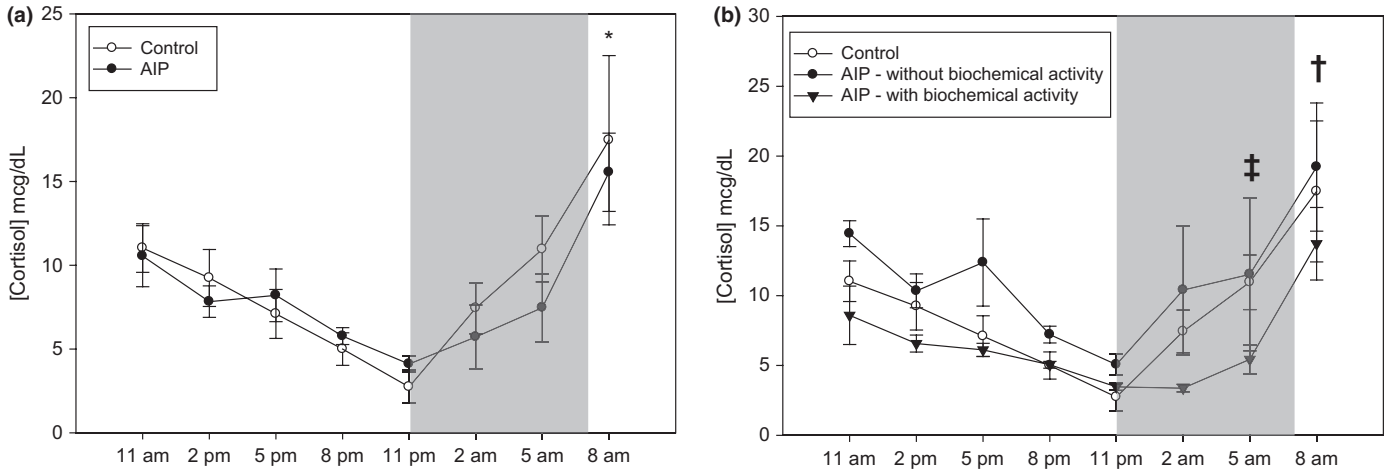

Figure 1 Time course of serum cortisol concentrations in subjects studied. Subjects had blood samples drawn and processed as described in Methods. (a) Concentrations of serum cortisol in control (n = 3) and all acute intermittent porphyria (AIP) subjects (n = 6). *Control group significantly different from all other time points within control except for 5 a.m., and AIP group significantly different from all other time points within AIP. (b) Concentrations of serum cortisol in control (n = 3) and AIP subjects with (n = 4) and without (n = 2) biochemical activity for time course studied. †AIP subjects with biochemical activity significantly different from other time points within group, AIP subjects without biochemical activity significantly different from 8 p.m. to 11 p.m. time points within group and control group significantly different from all other time points within control except 5 a.m. ‡AIP subjects with biochemical activity significantly different from control at same time point. Shaded areas represent times of usual sleep. Data are means ± SE.

cortisol in AIP subjects with biochemical activity showed a trend for lower serum cortisol levels in the early morning period, which was significantly lower in these subjects than in controls at 5 a.m. (Fig. 1b). Furthermore, total serum cortisol through the entire 21-h study period, as measured by AUC analysis, was significantly lower in AIP subjects with biochemical activity (124·47 ± 9·8 mcg/dL) than in control subjects (168·78 ± 21·7 mcg/dL). Individual subject serum cortisol levels are shown in Figure S1A. These results suggest that AIP subjects with biochemical activity have decreased levels of serum cortisol and blunted early morning increases in this analyte.

To determine if serum cortisol in AIP subjects demonstrates altered circadian oscillation, we performed single cosinor analysis on serum cortisol levels for all subjects, by the methods of Refinetti *et al.* [41]. While 2 of the 3 control subjects showed significant circadian oscillation for serum cortisol, only 2 of the 6 AIP subjects showed significant circadian oscillations (data not shown). We next performed single cosinor analysis on mean serum cortisol in control subjects, which showed significant circadian oscillations (Table S1). In contrast, cosinor analysis on mean levels in AIP subjects did not show significant circadian oscillations. Of importance, AIP subjects excreting normal concentrations of PBG (< 4·0 μM) showed significant circadian oscillations of serum cortisol, whereas clinically active AIP subjects ([urinary PBG]> 4·0 μM) did not (Table S1). In sum, these results indicate that biochemically active AIP subjects have blunted early morning increases and impaired circadian oscillations of serum cortisol.

Time course and levels of serum melatonin

For both control and AIP subjects, serum melatonin showed a trend of peaking during the night-time hour, with AIP subjects having significantly higher plasma melatonin at 5 a.m. than during daytime hours (Fig. 2a). At all time points studied, save one (8 p.m.), the mean values of serum melatonin in AIP subjects with biochemical activity were lower than for the AIP subjects without activity. However, the differences were relatively small and were not significant at the 5% level (Fig. 2b). Total AUCs for serum melatonin over the 21-h time period were also not different among subject groups (data not shown). Serum melatonin levels for all subjects are shown in Figure S1B.

To determine if serum melatonin in AIP subjects demonstrates altered circadian oscillation, we performed single cosinor analysis on serum melatonin levels of individual subjects. All 3 control subjects showed serum melatonin circadian oscillations, while 5 of 6 AIP subjects showed significant oscillations (data not shown). Circadian oscillations for average serum melatonin were also confirmed in control and AIP groups, as well as in AIP subgroups (Table S1). In sum, we did not find evidence for altered serum melatonin between control and AIP subjects, nor subgroups of AIP subjects.

Time course and levels of plasma ALA & PBG

Subjects with biochemically active AIP showed higher levels of PBG in plasma at all time points studied (Fig. 3b). In contrast, mean serum concentrations of ALA were similar in controls and subjects with AIP, and among the latter, there

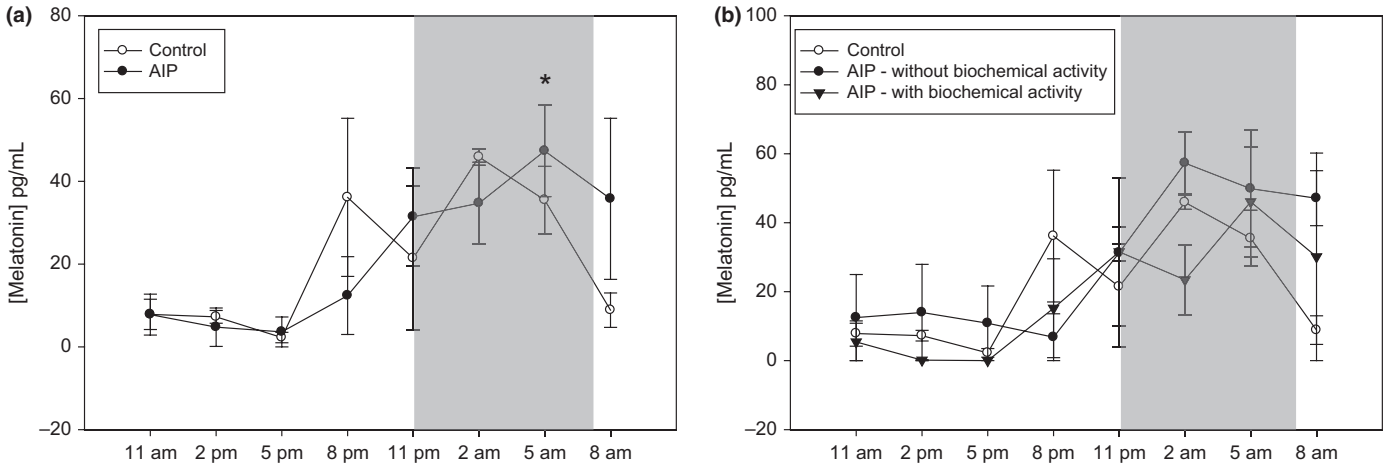

Figure 2 Time course of serum melatonin concentrations in subjects studied. Subjects had blood samples drawn and processed as described in Methods. (a) Concentrations of serum melatonin in control (n = 3) and all acute intermittent porphyria (AIP) subjects (n = 6). *mean value for AIP group at 5 a.m. significantly greater than mean values for this at 11 a.m., 2 p.m. and 5 p.m. time points. (b) Concentrations of serum melatonin in control (n = 3) and AIP subjects with (n = 4) and without (n = 2) biochemical activity. Shaded areas represent times of usual sleep. Data are means ± SE.

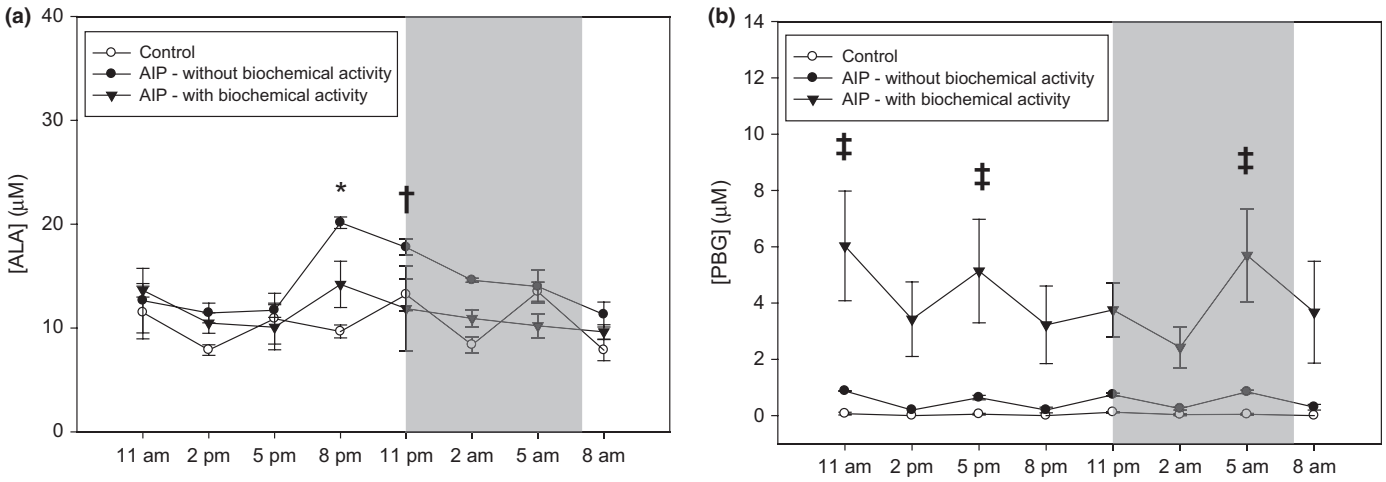

Figure 3 Time course of plasma 5-aminolevulinic acid (ALA) and porphobilinogen (PBG) concentrations in subjects studied. Subjects had blood samples drawn and processed as described in Methods. Mean concentrations of plasma (a) ALA and (b) PBG in control (n = 3) and acute intermittent porphyria (AIP) subjects with (n = 4) and without (n = 2) biochemical activity. *All groups significantly different from each other at 8 p.m. time point. †AIP subjects without biochemical activity significantly different from other subject groups within time point. ‡AIP subjects with biochemical activity significantly different from other subject groups within time point. Shaded areas represent times of usual sleep. Data are means ± SE.

were no differences between the subgroup with vs. without biochemical activity (Fig. 3a). Similarly, there were no differences in AUC over the 21-h study period (data not shown).

Total serum PBG concentration over the course of the experiment, as determined by AUC analysis, was significantly higher in AIP subjects with activity (84·8 ± 15·1 μM) than in AIP subjects without activity (10·4 ± 0·8 μM) or controls (0·757 ± 0·2 μM). There was also a strong trend for total serum PBG concentration over the course of the experiment to be higher for AIP subjects than for control subjects (P = 0·064). Although there were no significant circadian oscillations as determined by cosinor analysis in serum PBG concentration for any subject (data not shown), of considerable interest, the levels in subjects with active AIP showed a sine wave type of pattern with a period of approximately 6 h (Figure S2B). In addition, serum PBG levels in the AIP subjects without biochemical activity also showed a similar sine wave pattern, albeit with blunted amplitude (Figure S2B).

Urinary and plasma porphyrin concentrations at baseline

Average urinary porphyrin concentrations in spot urine samples taken at baseline are shown in Figure S3. AIP subjects showed a strong trend for an approximately 1–2 orders of magnitude increase in urinary uroporphyrin and 4-coproporphyrin-I and -III, although these trends were not significant, perhaps related to our small sample sizes (n = 2 for each group). Plasma porphyrin concentrations were undetectable (< 25 nM or < approximately 0·14 mcg/dL, the lower limit of detection for the UPLC/FLD assay used).

Expression of clock-controlled genes and selected genes of haem synthesis

We also investigated whether AIP subjects had altered expression of genes involved in circadian function by quantifying mRNA levels of selected clock-controlled genes in PBMCs collected at 11 a.m., 5 p.m., 11 p.m. and 5 a.m. *PER2* expression was significantly increased at 5 a.m. (Fig. 4a), and *CRY1* expression was increased at 11 p.m. (Fig. 4b), although neither gene showed differences between control and AIP subgroups at individual time points. Expression of *NR1D1*, the gene that encodes Rev-erb alpha protein, did not change significantly over time and was not significantly different between subject groups at any time point (Fig. 4c).

Because intracellular haem levels have been shown to oscillate in a circadian pattern [43], we investigated whether expression patterns of selected genes of haem synthesis showed circadian variability in control and AIP subjects. As shown in Fig. 5, all three such genes studied (*ALAS1*, *ALAS2* and *PBGD*) showed a strong trend for increased expression at 11 p.m. over control in AIP subjects with biochemical activity, with two of these genes, *ALAS2* and *PBGD*, significantly increased (P < 0·05).

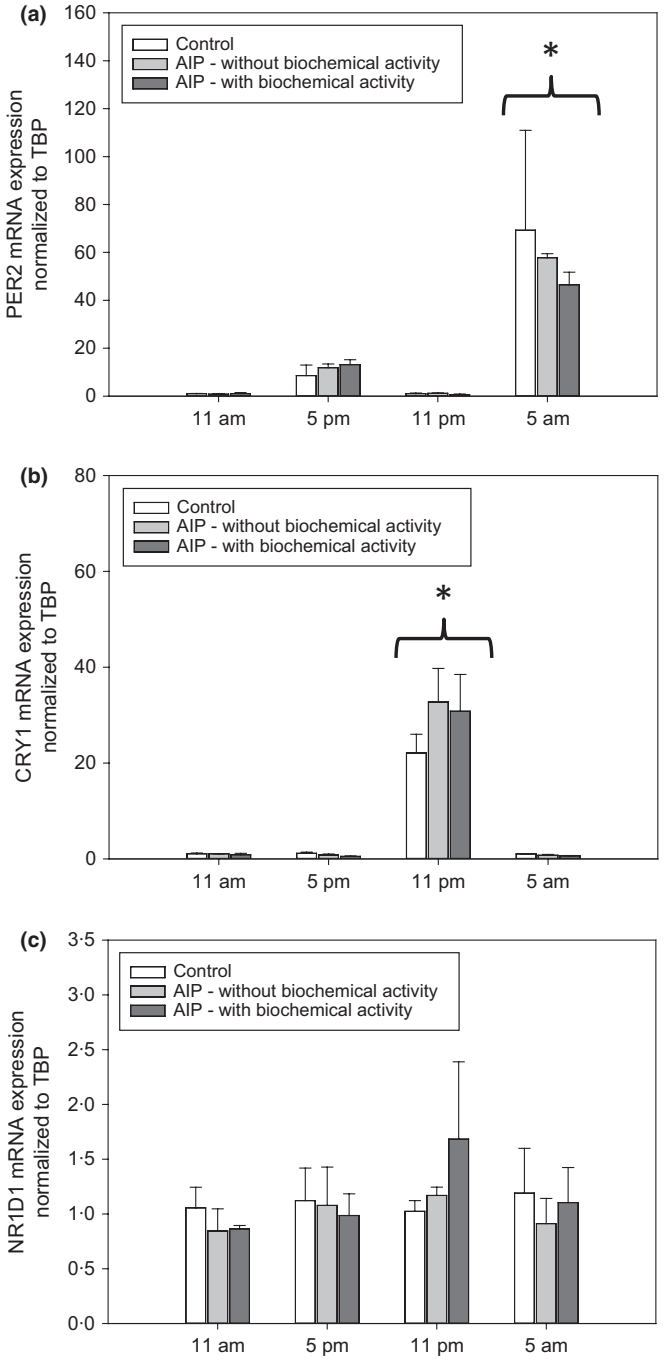

Figure 4 Selected clock-controlled gene mRNA expression in peripheral blood mononuclear cells (PBMCs) in subjects studied. Subjects had blood samples drawn and processed as described in Methods. mRNA expression for (a) PER2, (b) CRY1 and (c) NR1D1 using $\Delta\Delta C_t$ method in PBMCs for control (n = 3) and AIP subjects with (n = 4) and without (n = 2) biochemical activity. *Significantly different from other time points within respective subject group. Data are means ± SE.

Discussion

The major findings of this pilot study are that (i) asymptomatic AIP subjects with biochemical activity, defined as increased urinary and plasma levels of PBG, have lower levels of serum cortisol than normal controls or AIP subjects without biochemical activity, and they also exhibit significantly blunted increases in these levels in the morning (5 a.m., 8 a.m.; Fig. 1b). (ii) AIP subjects with biochemical activity show no increases in serum ALA but do show clear increases and striking sine wave fluctuations in plasma PBG throughout the course of the day (Figure S2). (iii) Overall, however, there is not a major abnormality in circadian oscillations of serum cortisol, melatonin, ALA or PBG in postmenopausal women with asymptomatic AIP. (iv) There are no abnormalities or differences in circadian expression of selected clock-controlled genes (*PER2, CRY1, NR1D1*) nor of genes of haem synthesis (*ALAS1, ALAS2, PBGD*) in PBMCs of women with AIP, compared with controls.

Acute attacks of severe pain, usually abdominal, severe constipation, tachycardia and systemic arterial hypertension are the clinical hallmarks of the acute porphyrias: AIP, hereditary coproporphyria, variegate porphyria and ALA dehydratase porphyria [1–6]. AIP is the most severe form of the common heterozygous forms of acute porphyria and is most often associated with recurrent attacks, probably because the normal level of activity of hepatic PBG deaminase is the lowest among the enzymes of haem synthesis distal to ALA synthase [1,2,4–6]. In women of child-bearing age, such attacks sometimes recur monthly especially during the luteal phases of their menstrual cycles, at which time there is a burst of progesterone, a porphyrogenic female hormone, produced by the *corpus luteum*. Although the pathogenesis of acute porphyric attacks is complex and still understood incompletely, a *sine qua non* is derepression of hepatic ALA synthase-1, leading to sometimes massive over-production and over-excretion of ALA and PBG (more than 1 millimole/day). That the major neuromuscular toxin is ALA or a by-product of ALA (not PBG) is implied by the fact that similar clinical features also occur in lead poisoning and hereditary tyrosinemia, type 1, both of which similarly are characterized by marked over-excretion of ALA but not of PBG [1,2,4–6,11].

In addition to severe acute porphyric attacks, many subjects with acute porphyria suffer from disorders of mood and affect, difficulty sleeping, difficulty concentrating, depression and anxiety. Whether these manifestations are directly related to relative hepatic haem deficiency is currently unknown. Similarly, it is unclear whether the current treatment of choice for severe acute attacks, namely intravenous haem administration, can improve these symptoms [1,2,4–6,44]. Other medical conditions reported by the volunteer subjects (i.e. systemic arterial

hypertension, high cholesterol, recurrent urinary tract infections, restless legs syndrome and mild depression) are not known to directly influence the clock, although some clinical and epidemiological studies have established that neurologically associated disorders, such as marked depression and symptomatic restless legs syndrome, may be associated with clock dysfunction. In this study, subjects with depression that was more than mild, as assessed by the Beck Depression Inventory, were excluded.

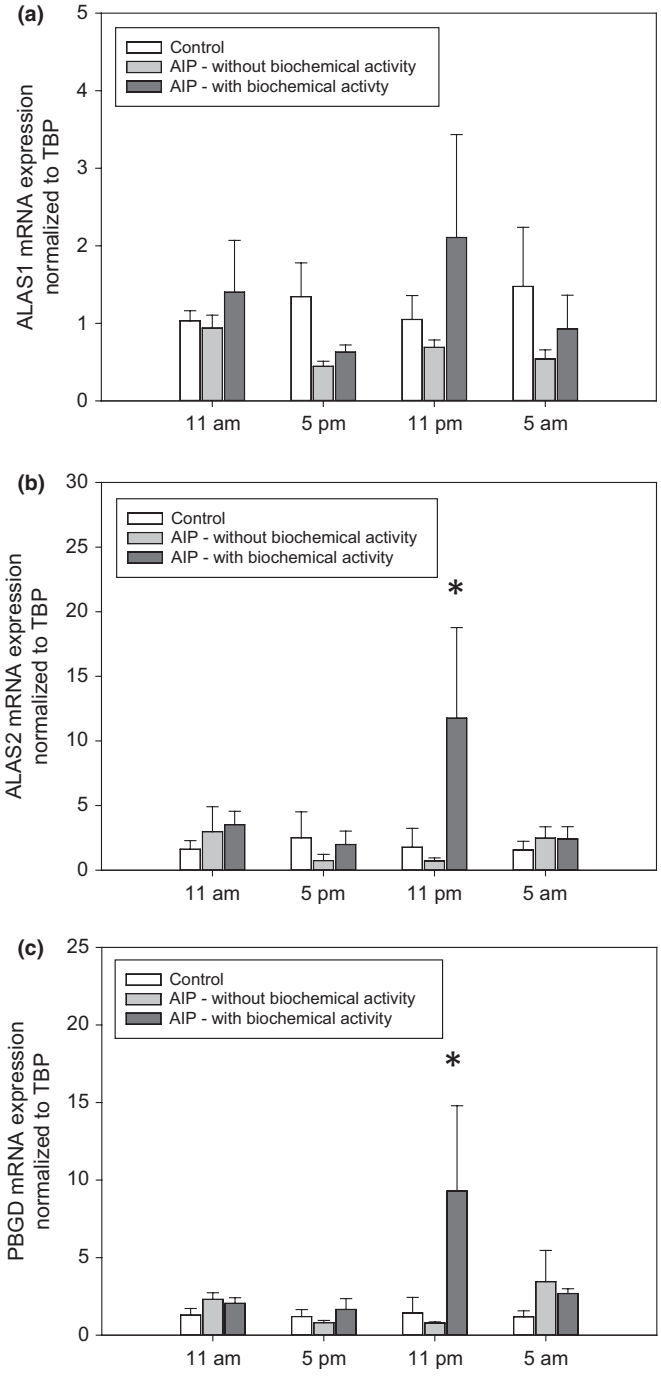

Figure 5 mRNA expression of selected genes involved in haem synthesis in peripheral blood mononuclear cells (PBMCs) in subjects studied. Subjects had blood samples drawn and processed as described in Methods. mRNA expression for (a) 5-aminolevulinic acid synthase-1 (ALAS1), (b) ALAS2 and (c) porphobilinogen deaminase (PBGD) using $\Delta\Delta C_t$ method in PBMCs for control (n = 3) and acute intermittent porphyria (AIP) subjects with (n = 4) and without (n = 2) biochemical activity. *AIP subjects with biochemical activity significantly different from other time points within group, and also different from other subject groups within same time point. Data are means ± SE.

Because of recent evidence linking haem to clock-controlled proteins and circadian rhythms [45], we hypothesized that subjects with biochemically active acute porphyria would prove to have abnormalities in their circadian rhythms. Although our results did not reveal major disturbances, they did show decreases in levels of total serum cortisol in AIP subjects with biochemical activity and, especially, a blunting of the normal circadian increase in these levels that normally occurs in the early morning (Fig. 1). They also showed a trend for decreased nocturnal levels of melatonin. The reasons behind these lower levels and blunted responses remain uncertain. Perhaps, such subjects have subtle defects in the hypothalamic–pituitary–adrenal axis, as has been described in severely symptomatic, biochemically active subjects with AIP [46]. Or, perhaps, these more subtle changes are due to mild relative haem deficiencies, leading to defects in clock-controlled genes and circadian rhythms [24,43,45,47].

Nocturnal levels of serum melatonin have been reported to be decreased in subjects with biochemically active, symptomatic AIP [16]. We did not find significantly different nocturnal levels of serum melatonin in subjects with asymptomatic AIP, some of whom showed no biochemical activity at the time of study (Fig. 2). However, the number of subjects studied here was small, and it may be that AIP subjects with greater biochemical activity and, presumably, greater defects in regulatory haem pools will show more striking differences from controls.

The synthesis of haem is under tight regulation, chiefly exerted at the level of ALA synthase, the first and normally rate-controlling enzyme of the pathway. Sufficiency or excess of haem leads to repression of ALAS1 mRNA levels [6,48–50] and to increased proteolytic cleavage of mitochondrial ALAS1 [51]. Oscillations in hepatic haem synthesis and in ALAS1 activity and ALA and PBG synthesis have long been known in rodent models of AIP [52–54]. The striking oscillations that we

observed in plasma levels of PBG (Figure S2B) suggest similar oscillations in humans with AIP.

Strengths of this study include the careful and thorough assessment of the subjects studied and the detailed characterization performed, including serum cortisol and melatonin and plasma ALA and PBG, and mRNA levels in PBMCs. Methods for analyses are state of the art.

Limitations of this study are that, due to limited funding, we were able to enrol only 10 subjects, and, unfortunately, due to very difficult peripheral venous access, among these, one control subject did not provide blood samples at most of the time points. Then, too, perhaps, it would have been preferred if subjects, prior to the day of study, had been able to spend several days in the sleep centre, becoming fully acclimated to it and more comfortable with the staff and surroundings. However, the costs of such a study design would have been prohibitively high, and the willingness of subjects to participate would have been much lower. Another limitation is that we were able to enrol only four subjects with biochemically active AIP, in whom one would expect more striking defects in regulatory haem pools. Three of these four had only slight increases in urinary PBG levels. It remains unknown whether AIP subjects with more marked biochemical activity will show greater abnormalities in serum cortisol or melatonin or plasma PBG levels than were observed here. Also, still unknown, but of obvious interest, is whether intravenous haem, the specific treatment of choice for full-blown attacks of acute porphyria [1–7], may also normalize serum cortisol or melatonin and/or ameliorate the difficulties in sleeping (Table 1) or myriad other 'minor' symptoms of which AIP subjects often complain.

In summary, in this pilot study, postmenopausal women with asymptomatic AIP with biochemical activity had lower levels of cortisol in the serum, a blunted early morning rise in serum cortisol and absence of normal circadian oscillations in these levels. In contrast, we did not find evidence of an abnormality of levels of serum melatonin or circadian oscillations in subjects with AIP not in crisis. No abnormalities in circadian rhythms or levels of clock-controlled genes or selected genes of haem synthesis were found in PBMCs, comparing AIP to control subjects.

Acknowledgements

This study was supported by the Avon Foundation and an NSF career award (to CVF), by grants and cooperative agreements from NIH (R01 DK38825; R15 HL 117199; U01 DK 0654201, U01 DK 065176, U01 DK 083027, U01 DK 065193 to HLB), by a grant from Carolinas HealthCare Foundation (to FRC), by institutional funds from Carolinas Medical Center and by an unrestricted gift from the American Porphyria Foundation. (Drs Caballes and Maddukuri are trainees supported by the APF.)

Address

Departments of Medicine, The Liver-Biliary-Pancreatic Center, Carolinas HealthCare System, Charlotte, NC, USA (S. Larion, F. R. Caballes, S.-I. Hwang, J.-G. Lee, W. E. Rossman, N. Steuerwald, T. Li, V. Maddukuri, G. Groseclose, H. L. Bonkovsky); Departments of Research, Carolinas HealthCare System, Charlotte, NC, USA (F. R. Caballes, V. Maddukuri); Carolinas HealthCare System, Charlotte, NC USA (S.-I. Hwang, J.-G. Lee, W. E. Rossman, J. Parsons, N. Steuerwald, T. Li, G. Groseclose, H. L. Bonkovsky); The Integrated Cellular Responses Laboratory, Department of Biological Sciences, Virginia Tech, Blacksburg, VA, USA (C. V. Finkielstein)

Correspondence to: Prof. Herbert L. Bonkovsky, Department of Medicine, Carolinas HealthCare System, 1000 Blythe Blvd, Charlotte, NC, 28236, USA. Tel.: 704 355 7516; fax: 704 355 7648; e-mail: herbert.bonkovsky@carolinas.org

Received 2 October 2012; accepted 1 April 2013

References

1 Bonkovsky HL. Porphyrin and heme metabolism and the porphyrias. In: Zakim D, Boyer TD, editors. *Hepatology: A Textbook of Liver Disease*. 2nd Edn. Philadelphia: Saunders; 1990; pp 378–424.
2 Hahn M, Bonkovsky HL. Disorders of porphyrin metabolism. In: Wu G, Israel J, editors. *Diseases of the Liver and Bile Ducts: A Practical Guide to Diagnosis and Treatment*. Totowa, NJ: Humana Press, 1998; pp 249–72.
3 Anderson KE, Bloomer JR, Bonkovsky HL *et al.* Recommendations for the diagnosis and treatment of the acute porphyrias. *Ann Intern Med* 2005;**142**:439–50.
4 Desnick R, Bishop DF, , Sassa S, , Anderson KE. Disorders of heme biosynthesis: X-linked sideroblastic anemia and the porphyrias. In: *Online Metabolic and Molecular Bases of Inherited Disease*. January 2006.Available at: http://www.ommbid.com/. Accessed on 28 March 2011.
5 Reichheld JR, Bonkovsky HL. The porphyrias, alpha-1 antitrypsin deficiency, cystic fibrosis, and other metabolic disorders of the liver. In: Bacon BR, O'Grady J, Di Bisceglie AM, Lake JR, editors. *Comprehensive Clinical Hepatology*. 2nd Edn. London, UK: Elsevier-Mosby Publishing, 2006; pp 369–96.
6 Bonkovsky HL, Hou W, Li T, Guo J-T, Narang T, Thapar M. Porphyrin and heme metabolism and the porphyrias. In: Wolkoff A, Lu S, Omary B editors. *Compr Physiol* 2013; **3**:1–37, The American Physiological Society. Bethesda, MD: Wiley and Co.
7 Bonkovsky HL, Healey JF, Lourie AN, Gerron GG. Intravenous heme-albumin in acute intermittent porphyria: evidence for repletion of hepatic hemoproteins and regulatory heme pools. *Am J Gastroenterol* 1991;**86**:1050–6.
8 Soonawalla ZF, Orug T, Badminton MN *et al.* Liver transplantation as a cure for acute intermittent porphyria. *Lancet* 2004;**363**:705–6.
9 Wahlin S, Harper P, Sardh E *et al.* Combined liver and kidney transplantation in acute intermittent porphyria. *Transpl Int* 2010;**23**: e18–21.
10 Dowman JK, Gunson BK, Mirza DF *et al.* Liver transplantation for acute intermittent porphyria is complicated by a high rate of hepatic artery thrombosis. *Liver Transpl* 2012;**18**:195–200.

11 Davis JR, Abrahams RH, Fishbein WI, Fabrega EA. Urinary delta-aminolevulinic acid (ALA) levels in lead poisoning. II. Correlation of ALA values with clinical findings in 250 children with suspected lead ingestion. *Arch Environ Health* 1968;**17**:164–71.
12 Tomokuni K, Ichiba M, Hirai Y. Elevated urinary excretion of beta-aminoisobutyric acid and delta-aminolevulinic acid (ALA) and the inhibition of ALA-synthase and ALA-dehydratase activities in both liver and kidney in mice exposed to lead. *Toxicol Lett* 1991;**59**:169–73.
13 Crimlisk HL. The little imitator–porphyria: a neuropsychiatric disorder. *J Neurol Neurosurg Psychiatry* 1997;**62**:319–28.
14 Anderson KE, Spitz IM, Bardin CW, Kappas A. A gonadotropin releasing hormone analogue prevents cyclical attacks of porphyria. *Arch Intern Med* 1990;**150**:1469–74.
15 Dollins AB, Zhdanova IV, Wurtman RJ, Lynch HJ, Deng MH. Effect of inducing nocturnal serum melatonin concentrations in daytime on sleep, mood, body temperature, and performance. *Proc Natl Acad Sci USA* 1994;**91**:1824–8.
16 Puy H, Deybach JC, Baudry P *et al.* Decreased nocturnal plasma melatonin levels in patients with recurrent acute intermittent porphyria attacks. *Life Sci* 1993;**53**:621–7.
17 Rajaratnam SM, Arendt J. Health in a 24-h society. *Lancet* 2001;**358**:999–1005.
18 Toh KL, Jones CR, He Y *et al.* An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* 2001;**291**:1040–3.
19 Mormont MC, Levi F. Circadian-system alterations during cancer processes: a review. *Int J Cancer* 1997;**70**:241–7.
20 Roenneberg T, Lucas RJ. Light, endocrine systems, and cancer–a view from circadian biologists. *Neuro Endocrinol Lett* 2002;**23**(Suppl 2):82–3.
21 Mirick DK, Davis S. Melatonin as a biomarker of circadian dysregulation. *Cancer Epidemiol Biomarkers Prev* 2008;**17**:3306–13.
22 Shechter A, Boivin DB. Sleep, hormones, and circadian rhythms throughout the menstrual cycle in healthy women and women with premenstrual dysphoric disorder. *Int J Endocrinol* 2010;**2010**:259345.
23 Rubio MF, Agostino PV, Ferreyra GA, Golombek DA. Circadian heme oxygenase activity in the hamster suprachiasmatic nuclei. *Neurosci Lett* 2003;**353**:9–12.
24 Kaasik K, Lee CC. Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. *Nature* 2004;**430**:467–71.
25 Padmanaban G, Venkateswar V, Rangarajan PN. Haem as a multifunctional regulator. *Trends Biochem Sci* 1989;**14**:492–6.
26 Ponka P. Cell biology of heme. *Am J Med Sci* 1999;**318**:241–56.
27 Yang J, Kim KD, Lucas A *et al.* A novel heme-regulatory motif mediates heme-dependent degradation of the circadian factor period 2. *Mol Cell Biol* 2008;**28**:4697–711.
28 Dioum EM, Rutter J, Tuckerman JR *et al.* NPAS2: a gas-responsive transcription factor. *Science* 2002;**298**:2385–7.
29 Zheng B, Albrecht U, Kaasik K *et al.* Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell* 2001;**105**:683–94.
30 Pandi-Perumal SR, Smits M, Spence W *et al.* Dim light melatonin onset (DLMO): a tool for the analysis of circadian phase in human sleep and chronobiological disorders. *Prog Neuropsychopharmacol Biol Psychiatry* 2007;**31**:1–11.
31 Hofstra WA, de Weerd AW. How to assess circadian rhythm in humans: a review of literature. *Epilepsy Behav* 2008;**13**:438–44.
32 Benloucif S, Burgess HJ, Klerman EB *et al.* Measuring melatonin in humans. *J Clin Sleep Med* 2008;**4**:66–9.
33 Selmaoui B, Touitou Y. Reproducibility of the circadian rhythms of serum cortisol and melatonin in healthy subjects: a study of three different 24-h cycles over six weeks. *Life Sci* 2003;**73**:3339–49.
34 Brainard GC, Hanifin JP, Greeson JM *et al.* Action spectrum for melatonin regulation in humans: evidence for a novel circadian photoreceptor. *J Neurosci* 2001;**21**:6405–12.
35 Danton M, Lim CK. Porphyrin profiles in blood, urine and faeces by HPLC/electrospray ionization tandem mass spectrometry. *Biomed Chromatogr* 2006;**20**:612–21.
36 Zhang J, Yasuda M, Desnick RJ *et al.* A LC-MS/MS method for the specific, sensitive, and simultaneous quantification of 5-aminolevulinic acid and porphobilinogen. *J Chromatogr B Analyt Technol Biomed Life Sci* 2011;**879**:2389–96.
37 Perotti C, Fukuda H, DiVenosa G *et al.* Porphyrin synthesis from ALA derivatives for photodynamic therapy. In vitro and in vivo studies. *Br J Cancer* 2004;**90**:1660–5.
38 Gederaas OA, Berg K, Romslo I. A comparative study of normal and reverse phase high pressure liquid chromatography for analysis of porphyrins accumulated after 5-aminolaevulinic acid treatment of colon adenocarcinoma cells. *Cancer Lett* 2000;**150**:205–13.
39 Murphy BA, Martin AM, Furney P, Elliott JA. Absence of a serum melatonin rhythm under acutely extended darkness in the horse. *J Circadian Rhythms* 2011;**9**:3.
40 Fernandez JR, Hermida RC, Mojon A. Chronobiological analysis techniques. Application to blood pressure. *Philos Transact A Math Phys Eng Sci* 2009;**367**:431–45.
41 Refinetti R. Non-parametric procedures for the determination of phase markers of circadian rhythms. *Int J Biomed Comput* 1992;**30**:49–56.
42 Nelson W, Tong YL, Lee JK, Halberg F. Methods for cosinor-rhythmometry. *Chronobiologia* 1979;**6**:305–23.
43 Rogers PM, Ying L, Burris TP. Relationship between circadian oscillations of Rev-erb alpha expression and intracellular levels of its ligand, heme. *Biochem Biophys Res Commun* 2008;**368**:955–8.
44 Bonkowsky HL, Tschudy DP, Collins A *et al.* Repression of the overproduction of porphyrin precursors in acute intermittent porphyria by intravenous infusions of hematin. *Proc Natl Acad Sci USA* 1971;**68**:2725–9.
45 Guenthner CJ, Bickar D, Harrington ME. Heme reversibly damps PERIOD2 rhythms in mouse suprachiasmatic nucleus explants. *Neuroscience* 2009;**164**:832–41.
46 Perlroth MG, Tschudy DP, Marver HS *et al.* Acute intermittent porphyria. New morphologic and biochemical findings. *Am J Med* 1966;**41**:149–62.
47 Kitanishi K, Igarashi J, Hayasaka K *et al.* Heme-binding characteristics of the isolated PAS-A domain of mouse Per2, a transcriptional regulatory factor associated with circadian rhythms. *Biochemistry* 2008;**47**:6157–68.
48 Drew PD, Ades IZ. Regulation of the stability of chicken embryo liver delta-aminolevulinate synthase mRNA by hemin. *Biochem Biophys Res Commun* 1989;**162**:102–7.
49 Hamilton JW, Bement WJ, Sinclair PR *et al.* Heme regulates hepatic 5-aminolevulinate synthase mRNA expression by decreasing mRNA half-life and not by altering its rate of transcription. *Arch Biochem Biophys* 1991;**289**:387–92.
50 Cable EE, Gildemeister OS, Pepe JA *et al.* Hepatic 5-aminolevulinic acid synthase mRNA stability is modulated by inhibitors of heme biosynthesis and by metalloporphyrins. *Eur J Biochem* 1996;**240**:112–7.
51 Tian Q, Li T, Hou W *et al.* Lon peptidase 1 (LONP1)-dependent breakdown of mitochondrial 5-aminolevulinic acid synthase

protein by heme in human liver cells. *J Biol Chem* 2011;**286**: 26424–30.
52 Tschudy DP, Waxman A, Collins A. Oscillations of hepatic delta-aminolevulinic acid synthetase produced by estrogen: a possible role of "rebound induction" in biological clock mechanisms. *Proc Natl Acad Sci USA* 1967;**58**:1944–8.
53 Marver HS, Collins A, Tschudy DP, Rechcigl M Jr. Delta-aminolevulinic acid synthetase. II. Induction in rat liver. *J Biol Chem* 1966;**241**:4323–9.
54 Waxman AD, Collins A, Tschudy DP. Oscillations of hepatic delta-aminolevulinic acid synthetase produced in vivo by heme. *Biochem Biophys Res Commun* 1966;**24**:675–83.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Time courses of serum (A) cortisol and (B) melatonin for individual study subjects.

Figure S2. Time courses of plasma (A) ALA and (B) PBG for individual study subjects.

Figure S3. Urinary porphyrin concentrations in subjects studied.

Table S1. Summary of variables in cosinor analysis.