Acute intermittent porphyria causes hepatic mitochondrial energetic failure in a mouse model

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A B S T R A C T

Acute intermittent porphyria (AIP), an inherited hepatic disorder, is due to a defect of hydroxymethylbilane synthase (HMBS), an enzyme involved in heme biosynthesis. AIP is characterized by recurrent, life-threatening attacks at least partly due to the increased hepatic production of 5-aminolaevulinic acid (ALA). Both the mitochondrial enzyme, ALA synthase (ALAS) 1, involved in the first step of heme biosynthesis, which is closely linked to mitochondrial bioenergetic pathways, and the promise of an ALAS1 siRNA hepatic therapy in humans, led us to investigate hepatic energetic metabolism in Hmbs KO mice treated with phenobarbital. The mitochondrial respiratory chain (RC) and the tricarboxylic acid (TCA) cycle were explored in the Hmbs −/− mouse model. RC and TCA cycle were significantly affected in comparison to controls in mice treated with phenobarbital with decreased activities of RC complexes I (−52%, p < 0.01), II (−50%, p < 0.01) and III (−55%, p < 0.05), and decreased activity of α-ketoglutarate dehydrogenase (−64%, p < 0.05), citrate synthase (−48%, p < 0.01) and succinate dehydrogenase (−53%, p < 0.05). Complex II-driven succinate respiration was also significantly affected. Most of these metabolic alterations were at least partially restored after the phenobarbital arrest and hepatic arginate administration. These results suggest a.cataplerosis of the TCA cycle induced by phenobarbital, caused by the massive withdrawal of succinyl-CoA by ALAS induction, such that the TCA cycle is unable to supply the reduced cofactors to the RC. This profound and reversible impact of AIP on mitochondrial energetic metabolism offers new insights into the beneficial effect of heme, glucose and ALAS1 siRNA treatments by limiting the cataplerosis of TCA cycle.

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1. Introduction

Acute intermittent porphyria (AIP, MIM #176000), the most common form of acute hepatic porphyrias, is due to mutations of hydroxymethylbilane synthase (HMBS, EC 2.5.1.61), the third enzyme in the heme biosynthetic pathway. AIP leads to the accumulation and increased urinary excretion of heme precursors such as 5-aminolaevulinc acid (ALA) and porphobilinogen (PBG) (Puy et al., 2010). This autosomal dominant disorder is responsible for life-threatening, neurovisceral symptoms including severe abdominal pain, nausea, vomiting, constipation or diarrhea, as well as peripheral neuropathy, tachycardia, high blood pressure, confusion, and seizure (Thadani et al., 2000).

The occurrence of AIP attacks frequently involves endocrine or environmental factors, such as infections, starvation, or the use of drugs, e.g. phenobarbital and sulfonamides. Hepatocytes require a variety of hemoproteins, including mitochondrial respiratory cytochromes and the cytochrome P450 enzymes (CYPs) that are involved in xenobiotic detoxification (Balwani and Desnick, 2012). Heme biosynthesis is regulated through negative feedback control by the heme itself on the first rate-limiting mitochondrial enzyme in the pathway, i.e. ALA synthase (ALAS). This enzyme catalyses the mitochondrial formation of ALA from the condensation of glycine and succinyl-CoA produced by the TCA cycle (Hunter and Ferreira, 2011). In the liver, heme regulates the synthesis and mitochondrial translocation of the ALAS1 housekeeping isomor (May et al., 1995). The ~50% residual activity of HMBS in AIP patients may be insufficient to handle the increased flux through the biosynthetic pathway, due to drug-induced AIP attacks, leading to the relative reduction of heme synthesis and accumulation of ALA/PBG (Puy et al., 2010). The subsequent sharp increase of ALAS1 activity may further enhance ALA accumulation. Treatment with heme arginate (Normosang®) probably represses ALAS1 induction through negative retro-control of heme itself at the transcriptional, post-transcriptional and post-translational levels (Thunell, 2006). Heme arginate may also decrease mitochondrial ALAS-1 protein through proteolysis (Tian et al., 2011). The pre-eminence of the liver in the pathophysiology of AIP and in ALA production has been highlighted by the spectacular benefit of liver transplantation in patients with severe AIP (Soonawalla et al., 2004; Dowman et al., 2012). In vitro, HepG2 cells in the presence of ALA are particularly sensitive to oxidative damage of mitochondrial and nuclear DNA (Onuki et al., 2004). In the rat, ALA, accumulates in the liver and diffuses to the body, acting as a neurotoxic agent and an endogenous pro-oxidant, leading to oxidative damage of tissues (Demasi et al., 1996; Sima et al., 1981).

Heme, the lack of which could in itself cause neurovisceral symptoms (Meyer et al., 1998), is partially synthesized in mitochondria (Nelson and Cox, 2000). Heme is a crucial cofactor of key oxidative phosphorylation (OXPHOS) components, such as ubiquinol-cytochrome c oxidoreductase (complex III, heme bl and bh), cytochrome oxidase (complex IV, heme a and a3) and cytochrome c, which transport electrons from complex III to complex IV. ALAS depends on the TCA cycle for its substrate, the succinyl-CoA and the erythroid ALAS2 has been shown to interact directly through a C-terminal specific binding domain with the β subunit of succinyl-CoA synthetase 2 (Bishop et al., 2012). In addition, the respiratory chain (RC) and ALA are major contributors of reactive oxygen species (ROS) that play a central role in the pathophysiology of AIP (Huang et al., 2011). The beneficial effect of intravenous glucose infusion in AIP crisis is mainly thought to be related to a direct down-regulation of the levels of ALAS1 messenger and protein (Oliveri et al., 2012). Moreover, this argues in favor of an energetic deficiency (Handschin et al., 2005) and it has been shown that the clinical expression of the disease is associated with a state of under-nutrition due to the sustained accumulation of ALA (Delaby et al., 2009). In a mouse model, the expression of the hepatic ALAS1 gene has been shown to be turned on by the fasting-induced transcriptional coactivator proliferator-activated receptor γ coactivator 1α (PGC-1α), one of the main regulators of energetic metabolism (Handschin et al., 2005; St-Pierre et al., 2006; Puigserver, 2005). The recent demonstration of the decrease in the insulin-like growth factor 1 (IGF-1) and transthyretin (prealbumin) in the serum of AIP patients (Delaby et al., 2009), together with the findings reported in a mouse model concerning the role of fasting in precipitating crises of acute porphyria (Handschin et al., 2005), reinforce the hypothesis that the nutritional and metabolic status of the liver may play a major role in the AIP clinical expression.

Despite the considerable links between heme biosynthesis and mitochondrial energetic metabolism, very few studies have addressed the possible involvement of a defect of mitochondrial energetic metabolism in AIP. The perspective of a subcutaneous RNAi therapeutic targeting of hepatic ALAS1 (http://www.alfylam.com/Programs-and-Pipeline/Alfylam-5x15/Porphyria.php) highlights the necessity of a better characterization of the ALAS1-RC relationship in hepatocytes. The aim of the present study was to examine the mitochondrial energetic profile in the liver of the Hmbs−/− deficient mouse model (Lindberg et al., 1996, 1999) during phenobarbital exposure and after heme arginate treatment.

2. Materials and methods

2.1. Animals

The murine Hmbs−/− model of AIP used in this study was generated by Lindberg et al. (1996, 1999). These mice are compound heterozygotes of two different disruptions (one null allele and a functional allele with a milder mutation) of Hmbs gene from the T1 strain C57BL/6-hmbstm1(geo)Jam and the T2 strain C57BL/6-hmbstm2(geo)Jam. These mice were shown to display a residual Hmbs activity of 30.9% in the liver.

Thirty-four female mice (8–10 weeks old) with the same genetic background, i.e. C57BL/6, were divided into four groups. The first group (16 mice), noted WT, consisted of wild type Hmbs+/+ mice used as controls. The second group (6 mice), noted BS, consisted of Hmbs−/− mice in the basal state. The third group (6 mice), noted PB, consisted of Hmbs−/− mice submitted to intraperitoneal injection of phenobarbital (Gardenal®) at 100 mg·kg−1 for 3 consecutive days as previously described (Johansson et al., 2003); these mice were sacrificed by cerebral dislocation 15 h after the last injection. The fourth group (6 mice) noted PB-HA, consisted of Hmbs−/− mice in a state of phenobarbital-induced AIP treated by an intraperitoneal injection of heme arginate (Normosang®) at 8 mg·kg−1 for 2 consecutive days; these mice were sacrificed by cerebral dislocation 2 h after the last injection. In humans, the recommended daily dose of heme arginate is 3 mg·kg−1·d−1 and is infused intravenously. Schuurmans et al. (2001) have shown that heme arginate at 3 mg·kg−1 (IP) did not significantly reduce plasma ALA concentrations in Hmbs−/− mice induced by phenobarbital injection (100 mg·kg−1, IP) and that the most effective dose was at least 6 mg·kg−1 (IP). Mice had access to food and water ad libitum. The metabolic profiles of urinary ALA and PBG, analyzed in the four groups of mice (Supplementary Fig. 1), confirmed the specific and reversible accumulation of ALA and PBG during exposure to phenobarbital. Urinary porphyrin precursor levels were determined on fresh urine collected before treatment and on each day of treatment (WT, BS and PB-HA groups of mice were sacrificed on the fifth day whereas the PB group of mice was sacrificed on the third day). ALA and PBG levels in pooled urine were analyzed by sequential ion-exchange chromatography using the ALA/PBG by Column Test Kit (Bio-Rad, CA, USA). All animal experiments were conducted...
in accordance with the Guide for the Care and Use of Laboratory Animals, eighth edition, 2011, National Research Council, National Academies Press, Washington, DC, USA.

2.2. Isolation of mitochondria from the liver

Mitochondria were isolated from the liver in the cold room (+4 °C) where all the reagents were kept on ice. Liver mitochondria were isolated using a modification of the differential centrifugation procedure described by Krähenbühl et al. (1994). The liver was cut into small pieces less than a millimeter cube in a buffer for isolation: 1 ml/100 mg liver composed of 100 mM saccharose, 50 mM KCl, 50 mM Tris–HCl, and 5 mM EGTA at pH 7.4. Then, it was homogenized with 7 up-and-down strokes in a 50 ml Potter at 1000 rpm. The homogenate was centrifuged at 600 × g for 10 min at +4 °C and the supernatant collected was filtered through a layer of gauze and centrifuged at 7000 × g for 10 min at +4 °C. The mitochondrial pellet was washed with the isolation buffer and centrifuged at 3500 × g for 10 min at +4 °C before being resuspended in a minute volume of isolation buffer and conserved on ice. The protein concentration was determined using the colorimetric bicinchoninic acid procedure (BC Assay Kit®, Montluçon, France) with BSA as standard.

2.3. Mitochondrial respiratory rates

Oxygen consumption was measured using Clark electrodes sensitive to oxygen. Isolated liver mitochondria were resuspended in the respiratory buffer containing in 120 mM KCl, 1 mM EGTA, 2 mM MgCl2, 3 mM HEPES, 5 mM KH2PO4, and 0.3% bovine serum albumin at pH 7.4. Respiratory rates were recorded at 37 °C in 1 ml glass chambers using a two-channel, high-resolution Oxygraph respiriometer (Oroboros, Innsbruck, Austria). Respirations (state II) were initiated in the presence of either complex I substrates (5 mM malate and 2.5 mM pyruvate), complex II substrates (10 mM succinate supplemented with 10 μM rotenone) or complex IV substrates (5 mM N,N,N,N′-tetramethyl-p-phenylenediamine reduced with 50 mM ascorbate). The active state of respiration (state III, coupled-respiration) was initiated by the addition of saturating ADP concentration (0.5 mM). Cyt c was added (8 μM) to check the outer mitochondrial membranes integrity. The ATP synthase inhibitor oligomycin was then added to obtain the state IV respiration rate. The maximal capacity of the electron transport system was recorded by uncoupling respiratory chain using 1 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). The respiratory control ratio (RCR) was defined as the State III/State IV using complex I substrates.

2.4. Mitochondrial enzyme activities

The activities of the mitochondrial OXPHOS complexes (I–IV) and of the TCA cycle enzymes in liver mitochondria were measured at 37 °C on a Beckman DU 640B spectrophotometer (Beckman Coulter, CA, USA) as described previously (Guillet et al., 2011).

2.5. Preparation of liver homogenates

All steps were performed in the cold room. Biopsies were weighed and rinsed quickly with an extraction buffer containing 220 mM mannitol, 75 mM saccharose, 10 mM Tris, 1 mM EGTA, pH 7.2. Samples were homogenized with 3 up-and-down strokes in a 2–3 ml Potter at 1000 rpm and centrifuged at 650 × g for 20 min. at 4 °C. The supernatant was conserved and the pellet was homogenized and centrifuged again. The two supernatants obtained were mixed and conserved at 4 °C.

2.6. Complex I (NADH ubiquinone reductase)

Complex I activity was measured in a reaction medium containing 50 mM phosphate buffer (pH 7.5), 3.75 mg ml⁻¹ fatty acid-free BSA, 0.1 mM decylubiquinone and 10 μg ml⁻¹ of mitochondrial proteins with, or without, 10 μM rotenone. After 2 min of incubation at 37 °C, the reaction was initiated by adding 0.1 mM NADH (final concentration). The enzyme activity was measured at 340 nm by monitoring the oxidation of NADH.

2.7. Complex II (succinate ubiquinone reductase)

Complex II activity was measured in a reaction medium containing 50 mM phosphate buffer (pH 7.5), 2.5 mg ml⁻¹ fatty acid-free BSA, 1 mM KCN, 5 μM rotenone, 5 μg ml⁻¹ antimycin, 30 mM succinate, 0.1 mM DCPIP and 5 μg ml⁻¹ of mitochondrial proteins. After 2 min of incubation at 37 °C, the reaction was initiated by adding 0.05 mM decylubiquinone. The enzyme activity was measured at 600 nm by monitoring the reduction of DCPIP.

2.8. Complex III (ubiquinone-cytochrome c reductase)

Complex III activity was determined by monitoring the reduction of cytochrome c at 550 nm. Five microgram of mitochondrial proteins were incubated for 30 s in 1 ml reaction medium consisting of 50 mM KH2PO4 (pH 7.5), 1 mg ml⁻¹ bovine serum albumin, 1 mM KCN, 100 μM oxidized cytochrome c and 50 μM EDTA, pH 7.5. The reaction was initiated by adding 100 μM decylubiquinol, and the optical density was measured for 1 min. The non-enzymatic reduction of cytochrome c was measured under the same conditions after addition of 10 μg ml⁻¹ antimycin A. The specific activity of complex III was calculated by subtracting the activity of the non-enzymatic reaction from that of the total activity.

2.9. Complex IV (cytochrome-c oxidase)

Complex IV activity was determined by monitoring the oxidation of cytochrome c at 550 nm. A 80 μM solution of reduced cytochrome c (92–97% reduced using dithionite) in 50 mM KH2PO4 (pH 7), was pre-incubated for 2 min at 37 °C. The reaction was initiated by adding 2 μg ml⁻¹ mitochondrial protein and the change in optical density at 550 nm was recorded for 1.5 min.

2.10. Citrate synthase

Citrate synthase activity was measured in a reaction medium containing 0.15 mM 5,5′-dithiobis(2-nitrobenzoic acid) regenerated with 1 M Tris buffer at pH 8.1, 0.5 mM oxaloacetate, 0.3 mM acetyl-CoA and 1% Triton X-100. After 2 min of incubation at 37 °C, the reaction was triggered by adding 50 μg ml⁻¹ of mitochondrial proteins, and the reduction of DNTB by CoASH was followed at 412 nm.

2.11. Fumarase

Fumarase activity was measured by monitoring the formation of fumarate from malate at 250 nm. The mix composed of 50 mM KH2PO4 (pH 7.4), 0.1 mM EDTA, 10 mM malate and 0.2% Triton was incubated for 5 min at 37 °C. The reaction was started by adding 50 μg ml⁻¹ (final volume) of mitochondrial proteins and the change in optical density at 250 nm was recorded for 4 min.

2.12. Succinate dehydrogenase (SDH)

Succinate dehydrogenase activity was measured after the reduction of 2,6-dichlorophenolindophenol (DCPIP) in the presence of 1 mM phenazine methosulfate (PMS) at 600 nm. Mitochondrial
proteins (50 μg ml⁻¹) were incubated in a mix containing 50 mM KH₂PO₄ (pH 7.5), 16 mM succinate, and 1.5 mM KCN for 5 min at 37 °C. The reaction was started by adding 100 μM DCPIP, and the optical density was recorded for 1.5 min.

2.13. Alpha-ketoglutarate dehydrogenase (α-KGDH), aconitase and isocitrate dehydrogenase synthase (ICDH)

The activities of three enzymes were measured during the same reaction using two different reaction mediums. First, to assay α-ketoglutarate dehydrogenase we used a mix containing 10 mM KH₂PO₄ (pH 7.2), 2 mM CaCl₂, 2 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton, 2 mM DTT, 2 mM α-ketoglutarate, 0.8 mM NAD⁺, 0.1 mM thiamine pyrophosphate (TPP) and 2 μg ml⁻¹ of mitochondrial protein. After 3 min of incubation at 37 °C, the reaction was triggered by adding 0.2 mM CoASH, and the reading was recorded for 2 min at 340 nm. Then, in the same cuvettes of the spectrophotometer, we added the second mix containing 10 mM citrate, 10 mM MgCl₂ and 0.8 mM NAD⁺ to assay aconitase for 4 min at 340 nm. Finally, we added 10 mM of isocitrate to assay isocitrate dehydrogenase, recording the reading for 3 min at 340 nm.

2.14. Statistical analysis

Statistical comparisons were made with the Mann-Whitney U test. Differences were considered statistically significant at p < 0.05. All analyses were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Mitochondrial respiration in the liver of phenobarbital treated Hmbs⁻/⁻ mice is altered

A spontaneous increase in state III respiration (the maximal phosphorylating respiration stimulated by ADP) driven by complex II was found in liver mitochondria from Hmbs⁻/⁻ mice in the basal state (BS) compared to wild type (WT) mice (+83%, p < 0.01) (Fig. 1B, right panel). There was no significant difference in the mitochondrial respiration driven by complex I substrates, malate and pyruvate, among the four groups of Hmbs⁻/⁻ mice studied, i.e. BS, WT, PB (mice with phenobarbital-induced AIP), and PB-HA (PB mice treated with heme arginate), in state II respiration, which is non-phosphorylating without ADP, and in state III respiration...
In contrast, the endogenous state II respiration (EII) driven by complex II, with succinate and rotenone as substrates, was significantly reduced in mitochondria from PB mice compared to BS mice (−59%, ′′′′ p < 0.05) (Fig. 1B). The endogenous ADP-stimulated state III respiration (EII) driven by complex II was also significantly reduced in liver mitochondria from PB mice compared to BS mice (−58%, ′′′′ p < 0.01), and in PB-HA mice the treatment significantly compensated the reduced respiration in PB mice (+58%, ′′′′ p < 0.05) (Fig. 1B). There were no significant differences among the four groups of mice with regard to complex IV (cytochrome oxidase)-driven mitochondrial respiration (Fig. 1C), nor were there any significant differences in the respiratory control ratios (RCRs).

Three control experiments showed that the mitochondrial respiratory inhibition was not due to the phenobarbital itself or to its derived products. Firstly, the phenobarbital directly administered in the Oxygraph to isolated liver mitochondria from WT Hmbs+/− mice did not affect the respiratory rate (data not shown). Secondly, WT mice injected with phenobarbital (WT-PB) showed no significant impairment of the mitochondrial respiration driven by complexes I and II or by the isolated complex IV in comparison to non-injected controls (WT) (Supplementary Fig. 2). Lastly, enzymatic activities of respiratory chain complexes, in liver of WT mice treated with phenobarbital, were not significantly affected (Supplementary Fig. 2).

3.2. The enzymatic activities of the first three mitochondrial respiratory chain complexes are altered in the liver of Hmbs+/− mice exposed to phenobarbital

Mitochondrial respiratory complex I activity was significantly reduced (Fig. 2) in the liver of PB mice compared to BS mice (−52%, ′′′′ p < 0.01) with a partial restoration of activity in the PB-HA mice (+39%, ′′′′ p < 0.05). Complex II activity was significantly reduced in the liver of PB mice compared to BS mice (−50%, ′′′′ p < 0.01) but without restoration of activity in the PB-HA mice. Complex III activity was significantly reduced in the liver of PB mice compared to BS mice (−55%, ′′′′ p < 0.05) with an almost complete restoration of activity in the treated PB-HA mice (+80%, ′′′′ p < 0.05). Complex IV hemic activity was not significantly different in the liver mitochondria of the four groups of mice studied.

The analysis of protein levels of the subunits of complexes I–V in liver homogenates, using an OXPHOS antibody cocktail, showed no significant differences in the mitochondria from PB mice compared to BS mice (Supplementary Fig. 3). Similarly, the expression levels of mitochondrial (MnSOD) and cytosolic (CuZnSOD) superoxide dismutases, which are usually stimulated in response to oxidative stress, showed no significant differences between PB mice and BS mice. These two results suggest that the reduced activity of RC complexes observed in AIP mice exposed to phenobarbital is due neither to reduced OXPHOS protein expression nor to increased ROS production (Supplementary Fig. 4).

3.3. The mitochondrial enzymatic activities of three TCA cycle enzymes are altered in the liver of Hmbs+/− mice exposed to phenobarbital

The mitochondrial enzymatic activities of five TCA cycle enzymes were investigated (Fig. 3): succinate dehydrogenase (SDH), fumarase, α-ketoglutarate dehydrogenase (α-KGDH), citrate synthase (CS) and isocitrate dehydrogenase (ICDH). After phenobarbital injection, the activities of α-KGDH, CS and SDH were reduced by 64% (′′′ p < 0.05), 48% (′′′′ p < 0.01) and 53% (′′′′ p < 0.05), respectively, in mitochondria from PB mice compared to BS mice, whereas the activities of fumarase and ICDH were not affected. The remission in the treated PB-HA mice completely compensated the α-KGDH defect (+244%, ′′′′ p < 0.05) and partially compensated the SDH defect (+30%, ′′′′ p < 0.05) but failed to compensate the CS defect. As in the case of most RC complex activities, the SDH and CS activities were spontaneously greater, (+55%, ′′′′ p < 0.05) and (+70%,
Few studies have addressed the question of the possible involvement of mitochondrial energetic defects in the pathophysiology of AIP. Pereira et al. (1992) showed that the chronic treatment of rats with ALA decreased citrate synthase activity in the liver and skeletal muscles, producing fatigue in the animals significantly earlier than in controls. Ogura et al. (2011) reported that ALA oral administration in mice led to a 1.5-fold increase of cytochrome c oxidase activity in the liver, accompanied by increased ATP production, suggesting that ALA may affect energy metabolism. Our study on an AIP mouse model reveals a failure of mitochondrial energetic metabolism in the liver after phenobarbital injection.

During the phenobarbital exposure, the RC and the TCA cycle of the liver mitochondria of the mice were both profoundly affected, and the treatment was accompanied by partial or complete recovery. Contrary to expectation, we found no defect specifically affecting the three RC components containing heme as the prosthetic group, i.e. complex III, complex IV and cytochrome c, which transmits electrons from complex III to complex IV. Indeed, although complex III was affected, complex IV was not since the normal respiration driven by complex I substrates ensured full cytochrome c functionality. In contrast, the activities of mitochondrial complexes I–III were greatly affected in the liver during the phenobarbital exposure, but the decreased activities were not due to reduced enzyme content. Complexes I and II are indeed highly regulated by post-translational modifications, particularly by phosphorylation, acetylation, succinylation and by allosteric regulation. Complex I and II are directly impacted by the available supplies from the Krebs cycle intermediates. For example, the Krebs cycle metabolic intermediate succinyl-CoA activates complex II (Kearney et al., 1972) and the acetylation state of complexes I and II is tightly regulated by sirtuin 3, the activity of which relies on the NAD⁺ level and the NADH/NAD⁺ ratio (Peek et al., 2013), this later ratio being greatly dependent on the Kreb’s cycle activity. The respiratory measurements revealed that complex II-driven respiration, which is usually privileged in hepatocytes,
was altered by the injection of phenobarbital and restored after heme treatment.

Complexes I–III all contain iron-sulfur clusters that could be the target of oxidative stress generated by the overproduction of ALA in AIP. Iron-sulfur clusters are small inorganic cofactors formed by the tetrahedral coordination of iron with the sulfur groups of amino acids residues. Such iron-sulfur clusters containing enzymes are known to represent critical targets for oxygen free radicals (Röig et al., 2002) that contribute to the pathophysiology of AIP (Hermes-Lima et al., 1992). However, in our model, none of the ROS cytotoxic (CuZnSOD) and mitochondrial (MnSOD) detoxifying enzymes, known to be sensitive markers of response to oxidative stress, were affected.

The second feature distinguishing the first three RC complexes from the others is that they all constitute points of entry for electrons in the RC. Thus, we hypothesized that a general lack of substrate supply (NADH for complex I and FADH2 for complex II) to the RC might explain the OXPHOS defect. We therefore explored the TCA cycle since complex II-driven respiration was found to be functionally affected and complex II activity (SDH) is shared by both the RC and the TCA cycle. Moreover, the heme biosynthesis pathway is closely linked to the TCA cycle that provides succinyl-CoA as a carbon source for the initial ALAS step of heme biosynthesis. We found that the TCA cycle was greatly affected at least at three levels, i.e., those of citrate synthase, the regulatory enzyme that orients the first step of the TCA flux and, interestingly, the two enzymes involved in the synthesis or utilization of succinyl-CoA, i.e., α-KGDH (one of the three regulatory enzymes of the TCA cycle) and SDH. The sharp increase of ALAS1 activity during the AIP crises probably consumes most of the succinyl-CoA available in the mitochondria. The acute subtraction of this intermediate metabolite could alter the metabolite flux through the TCA cycle, a phenomenon known as cataplerosis, which is usually compensated by anaplerotic reactions that counter the lack of the metabolite (Nelson and Cox, 2000). ALAS induction could also occur in Hmbs<sup>−/−</sup> WT mice treated with phenobarbital since a complex series of nuclear receptors and xenosensors control ALAS1 up-regulation upon exogenous invasion (Podvinec et al., 2004). However, Jover et al. (2000) showed that ALAS activity and mRNA were not significantly elevated in the liver by repeated phenobarbital administration in control mice whereas it was substantially induced in Hmbs<sup>−/−</sup> mice. Therefore, this absence of ALAS1 induction strongly supports the lack of cataplerosis and OXPHOS defect we found in the Hmbs<sup>−/−</sup> WT mice.

Our results, offering the first demonstration of a transitory implication of the mitochondrial RC and TCA cycle enzymes in the hepatic pathophysiology of AIP, are schematized in Fig. 4. The transfer of succinyl-CoA from the TCA cycle to ALA synthesis leads to cataplerosis such that the TCA cycle is unable to provide reduced substrates to the RC. The decreased supply of NADH and FADH2 to the RC reduces the activities of complexes I–III together with the complex II-driven respiration that is usually privileged in hepatocytes, thus leading to an energetic failure. These findings suggest a putative energetic beneficial role of siRNA directed against hepatic ALAS1 and are supported by the results of the two treatments that have proved efficacious in the management of AIP, i.e., heme arginate and carbohydrates. On one hand, heme arginate inhibits ALAS1, thus increasing the availability of succinyl-CoA for the TCA cycle and the NADH, FADH2 supply for the RC; on the other hand, the administration of carbohydrates increases the availability of acetyl-CoA for the TCA cycle by upregulating citrate synthase activity and thereby the metabolic flux across the TCA cycle. However, in...
addition to this hypothesis of cataplerosis and in regard to the tight interconnection of the metabolic pathways involved in this pathophysiological model of AIP, we cannot exclude the participation of other mechanisms either in the impairment of the RC or in mitochondrial metabolic disturbances. Nor can we exclude the direct or indirect impact of heme deficiency and ALA overproduction on respiration.

Our study also showed that the enzymatic activities of the RC and TCA cycle were spontaneously increased on the transgenic Hmbs−/− mice outside of the period phenobarbital exposure, suggesting the operation of a compensatory mechanism already present in the basal state but without any clinical manifestation. The mechanism restoring metabolic re-equilibrium in the transgenic mice might be compromised by phenobarbital. Indeed, an elevated blood lactate concentration, a classical hallmark of OXPHOS dysfunction, has been reported in 6 patients compared to controls outside the period of crises (Herrick et al., 1990), showing that AIP patients may be chronically affected by a subtle, subclinical OXPHOS defect.

Although the excess production of heme precursors in AIP is mainly of hepatic origin, the liver function is frequently preserved in patients and only a minority of them shows a mild transaminase increase. Thus, the OXPHOS impairment demonstrated in the present article may not have reached a threshold high enough to cause a severe liver failure.

The investigation of the impaired energetic metabolism of liver mitochondria in the AIP mouse model is prerequisite to the introduction of any new hepatic ALAS1 biotherapy for patients with the disease. Determining whether a similar mitochondrial dysfunction affects tissues and organs other than the liver and during the chronic phases of the disease should help to define a novel therapeutic strategy for the disorder.

Conflict of interest statement

No conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.03.032.

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