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Ursodesoxycholic Acid and Heme-Arginate are Unable to Improve Hematopoiesis and Liver Injury in an Erythropoietic Protoporphyria Mouse Model

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Summary

Erythropoietic protoporphyria (EPP) is an inherited disorder of heme biosynthesis caused by partial ferrochelatase deficiency, resulting in protoporphyrin overproduction which is responsible for painful skin photosensitivity. Chronic liver disease is the most severe complication of EPP, requiring liver transplantation in some patients. Data from a mouse model suggest that cytotoxic bile formation with high concentrations of bile salts and protoporphyrin may cause biliary fibrosis by damaging bile duct epithelium. In humans, cholestasis is a result of intracellular and canalicular precipitation of protoporphyrin. To limit liver damage two strategies may be considered: the first is to reduce protoporphyrin production and the second is to enhance protoporphyrin excretion. Bile salts are known to increase protoporphyrin excretion via the bile, while heme arginate is used to decrease the production of porphyrins in acute attacks of hepatic porphyrias. The Griseofulvin-induced protoporphyria mouse model has been used to study several aspects of human protoporphyria including the effects of bile salts. However, the best EPP animal model is an ethylnitrosourea-induced point mutation with fully recessive transmission, named ferrochelatase deficiency (*Fech^{m1Pas}*). Here we investigate the effect of early ursodesoxycholic acid (UDCA) administration and heme-arginate injections on the ferrochelatase deficient EPP mouse model. In this model UDCA administration and heme-arginate injections do not improve the protoporphyric condition of *Fech^{m1Pas}/Fech^{m1Pas}* mice.

Key words
Erythropoietic protoporphyria • Ursodesoxycholate • Heme • Mouse model

Abbreviations used (defined in text):
EPP – erythropoietic protoporphyria, PP – protoporphyrin, FECH – ferrochelatase, TBil – total serum bilirubin, ALP – serum alkaline phosphatase, ASAT – aspartate amino transferase, ALAT – alanin amino transferase, UDCA – ursodesoxycholic acid, HA – heme arginate

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Introduction

Erythropoietic protoporphyria (EPP) is an inherited disease of heme synthesis caused by a partial deficiency of the mitochondrial enzyme ferrochelatase (FECH; EC 4.99.1.1), which catalyses the insertion of ferrous iron into protoporphyrin IX (Anderson *et al.* 2001, Ferreira 1999). Cutaneous photosensitivity is the most common symptom of human protoporphyria (Lim 1989) but progressive functional deterioration and hepatocytes death associated with crystalline protoporphyrin deposition in Kuppfer cells, hepatocytes, biliary ducts and cirrhosis is the main dramatic complication of the disease. Severe hepatobiliary disease and hepatic failure are indications for orthotopic liver transplantation (Gross *et al.* 1998). However, less than 10 % of EPP allele carriers will develop symptoms and less than 2 % will develop severe hepatic failure. EPP has long been considered as an autosomal dominant disease with incomplete penetrance (Bloomer *et al.* 1976), despite two cases of purely recessive transmission (Lamoril *et al.* 1991, Sarkany *et al.* 1994). Patients show a range of phenotypic severity, which is not strictly correlated with the nature of the mutation (Gouya *et al.* 1998, Henriksson *et al.* 1996, Rufenacht *et al.* 1998). Recent data have shown that patients suffering from photodermatitis have reduced levels of FECH enzyme activity (15 to 30 % of normal) due to the co-inheritance of a null allele with a normal "low-expressed" allele (Gouya *et al.* 1996, Gouya *et al.* 1999, Gouya *et al.* 2002), but, at present, there is no reliable method for the early detection of those at high risk of severe hepatic failure. Recently, in one Japanese EPP family, it has been shown that hypermethylation of the wild-type ferrochelatase allele was closely associated with severe liver complications (Onaga *et al.* 2004). This was the first case reporting a genetic risk factor for hepatobiliary complications in EPP. However, there are no established guidelines to show the genetic risk factors that would identify patients with a high probability of developing severe liver disease, thus allowing for preventive care and treatment. Therefore, classical therapy is essential to avoid or reduce hepatobiliary complications of EPP.

Two EPP mouse models have been reported. A FECH exon 10 deletion was generated by gene targeting, resulting in a dominant-negative effect and embryonic lethality of homozygotes (Magness and Brenner 1999, Magness *et al.* 2002). Heterozygotes show mild protoporphyria with no liver disease. The best animal model is an ENU-induced point mutation named ferrochelatase deficiency (symbol *Fech*^{m1Pas}, *fech* thereafter). The mutation shows a fully recessive transmission (Tutois *et al.* 1991). A T to A substitution at position 293 replaces a methionine by a lysine at residue 98 (Boulechfar *et al.* 1993). In the BALB/cByJIco genetic background, to which the mutation was originally backcrossed, homozygotes show 5 % residual FECH activity in the liver and spleen, and develop skin lesions, jaundice and severe hepatic dysfunction with massive PP deposits. This model, which mimics the most severe forms of the disease, has been used to show that gene therapy and cellular therapy may dramatically improve the condition (Fontanellas *et al.* 2000, Fontanellas *et al.* 2001, Pawliuk *et al.* 1999, Richard *et al.* 2001, Richard *et al.* 2004). To our knowledge this model has never been used for therapeutic trials with ursodesoxycholic acid (UDCA) and heme arginate (HA). UDCA is known to stimulate bile secretion and counteract cholestasis (Paumgartner and Beuers 2004). In humans UDCA is used to dissolve cholesterol gallstones and treat biliary cirrhosis (Poupon *et al.* 1994, Fischer *et al.* 2004, Paumgartner and Beuers 2004). Hematin or HA (Tenhunen *et al.* 1987) is established in the treatment of acute attacks of hepatic porphyrias (Nordmann and Puy 2002), repressing ubiquitous 5-aminolevulinic acid synthase but not the erythroid-specific isoform of the enzyme, and thereby decreasing porphyrins production in the liver (Kappas *et al.* 1995). However HA has shown potential benefit in erythropoietic protoporphyria in which the bone marrow is the predominant site of porphyrins overproduction (Bloomer and Pierach 1982, Potter *et al.* 1996).

The aim of our study was to investigate the effect of early administration of UCDA and HA intraperitoneal injections on hematological and biochemical parameters used as indicators for the hepatobiliary and bone marrow functions of protoporphyric *fech/fech* mice.

Material and Methods

Animals

In the EPP murine model (Fech^{m1Pas}) the genetic defect is transmitted in an autosomal recessive fashion and consists of a single base pair change in the coding sequence of the murine Fech gene, resulting in marked reduction of the encoded enzyme catalytic activity. The original Fech^{m1Pas} mutation (Tutois *et al.* 1991) had been

previously backcrossed to the BALB/c inbred background for over ten generations. At each backcross generation, and in further crosses, mouse genotypes were identified by amplifying a genomic segment encompassing the point mutation, which removes a BspHI restriction site. PCR products were produced and digested as previously described (Boulechfar *et al.* 1993). All mice were housed in the same animal room throughout the study. They received unlimited, autoclaved water and irradiated food pellets. According to standard husbandry procedures, they were maintained in filter-top cages with artificial fluorescent light, under a 12-h light/dark cycle. Food for the experimental groups treated with UDCA was medicated with 0.5 %, 0.1 % or 0.02 % UDCA (Sigma Chemical Co., St. Louis, MO) by weight. UDCA-medicated pellets were given to both pregnant females and their pups until three months of age. Mice treated with HA (e.g. human hemin, Normosang®, Orphan Europe, Paris) were injected intraperitonealy with 3mg/kg HA every 3 days from weaning to three months of age.

Two series of mice were bred and analyzed. The first series included 5 groups (+/+ and fech/fech controls and fech/fech treated with 0.5 %, 0.1 % or 0.02 % UDCA) of three (0.5 % UDCA-treated mice), five (0.1 % and 0.02 % UDCA treated mice) and ten (control +/+ and fech/fech mice) 3-month-old mice which were analyzed for hematological and biochemical parameters. The second series included 3 groups (+/+, untreated f/f and HA-treated f/f) of ten 3-month-old mice which were analyzed for the same hematological and biochemical parameters.

Hematology and biochemistry

Mice were anaesthetized by intraperitoneal injection with a xylazine/ketamine mixture and weighed. Blood was collected by retroorbital sinus puncture. Red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), mean cell volume (MCV), and mean cell content in Hb (MCCH) were measured using a SCIL Vet'ABC® counter (SCIL GmbH, Viernheim, Germany). Total serum bilirubin (TBil), serum alkaline phosphatase (ALP), and serum aminotransferases (ASAT and ALAT) were measured with a VetTest® analyzer (IDEXX, Cergy-Pontoise, France).

RBC fluorescence was used to evaluate erythrocytic PP concentration, and was measured by flow cytometry using a Facscan® analyzer (Amersham). Total blood was diluted 1:15 with 0.9 % NaCl. Geometric mean of RBC fluorescence was measured using FL3 channel. A 3-month-old C57BL/6JIco +/+ mouse was used to calibrate the analyzer; this value was substracted from the value of every other mouse tested.

Statistical analysis

One-way ANOVA were performed with StatView F-4.1 software (Abacus Concepts, Berkeley, CA). Distribution of values was assessed for normality by comparison with a normal distribution with the same mean and standard deviation using a Kolmogorov-Smirnov test. To reach distribution normality, logarithmic transformation of biochemical parameters (RBC fluorescence, TBil, ALP, ASAT and ALAT) was used for ANOVA.

Results

Effect of HA injections

HA-treated or untreated *fech/fech* homozygotes showed growth retardation compared to +/+ mice at 3 weeks of age (data not shown). At 3 months of age, despite the severe hepato- and splenomegaly observed in all *fech/fech* mice resulting in abdomen enlargement, body weight was reduced compared to +/+ mice. HA-treated *fech/fech* mice showed no difference in body weight compared to untreated *fech/fech* mice. Serum and urine were icteric in *fech/fech* mice, treated or not. Jaundice was visible on ears of all *fech/fech* mice. Total serum bilirubin (TBil) was highly increased in the two groups compared to +/+ mice (Fig. 1). Moreover, TBil was higher in HA-treated *fech/fech* mice than in untreated *fech/fech* mice ($p=0.009$).

Hemoglobin content (Hb) and hematocrit (Ht) were not different between HA-treated and untreated *fech/fech* mice but were very significantly reduced compared with +/+ mice ($p<0.0001$ for each group of *fech/fech* mice compared to +/+ mice). On the other hand, red blood cells count (RBC) was statistically different between the two groups of *fech/fech* mice ($p=0.014$), but not statistically different between HA-treated *fech/fech* and +/+ mice.

Red blood cells fluorescence (RBC fluo) was measured as an estimate of intraerythrocytic accumulation of protoporphyrin. All *fech/fech* mice had much higher levels of RBC fluo than +/+ ice, but HA-treated mice had even higher levels than untreated *fech/fech* mice ($p<0.0001$), resulting from dramatically high accumulation of protoporphyrin in RBC.

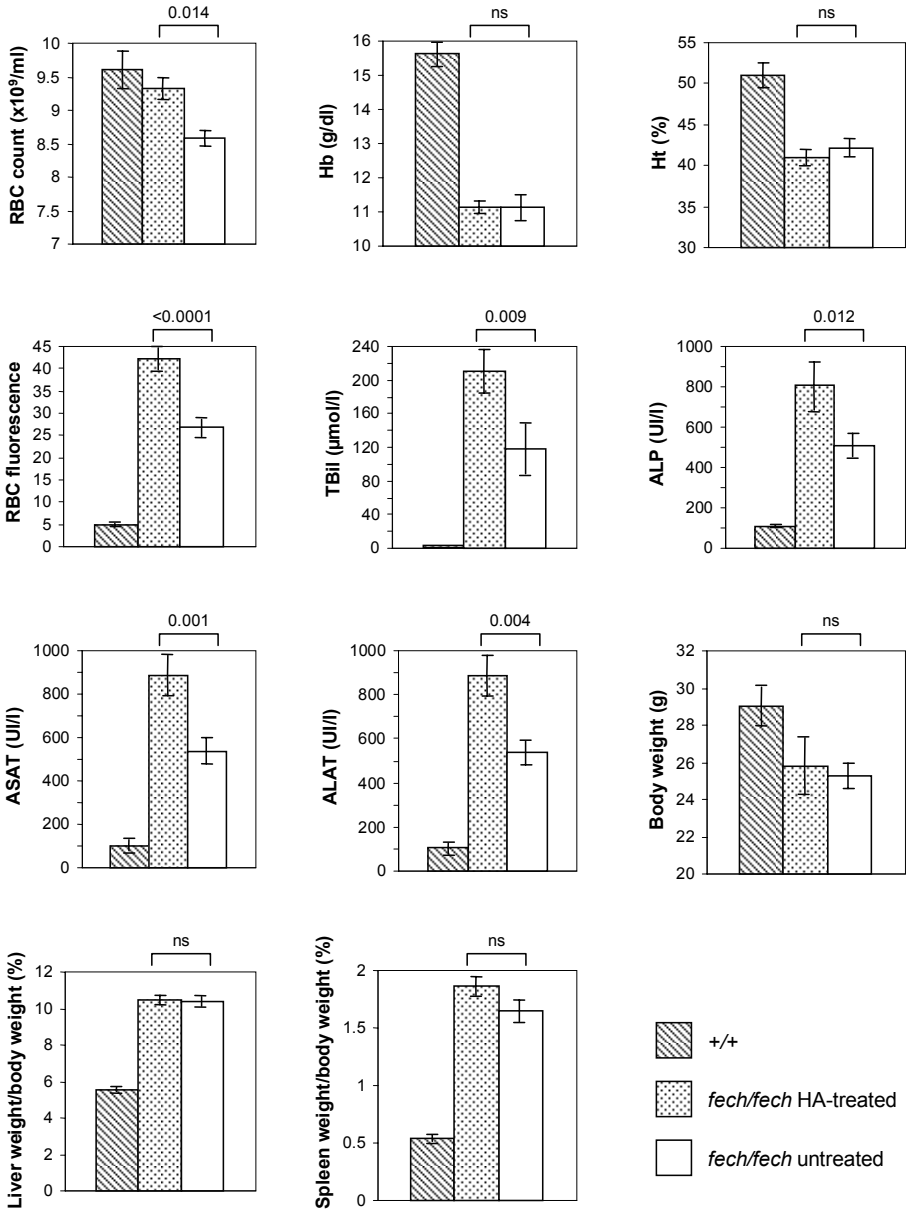

Fig. 1. Biological parameters measured in groups of ten 3-month-old +/+, *fech/fech* and *fech/fech* HA-injected mice. Data shown as mean ± sem. On the top of each graph is shown the p-value of the comparison between HA-treated and untreated *fech/fech* mice.

Hepatobiliary function was assesed by measuring enzymes which reflect hepatocyte (ASAT, ALAT) and biliary duct cell (ALP) damage. All *fech/fech* mice showed very high levels of these three enzymes compared to +/+ mice (Fig. 1) and HA-treated *fech/fech* mice showed the highest values of the three group.

Effect of UDCA administration

UDCA-medicated food pellets were given to *+/fech* heterozygous breeding pairs. Their pups were genotyped and received also medicated food from birth to 3 months of age. The first group of mice was treated with 0.5 % UDCA. Adult mice showed a dramatic reduction of fertility and all pups showed important growth retardation. *fech/fech* pups were very icteric. Only three *fech/fech* mice were analysed at 3 months of age, despite very poor condition (data not shown). In further experiments, doses of 0.1 % and 0.02 % were used.

When compared to untreated *fech/fech* mice, homozygous mice treated with 0.1 % UDCA showed no apparent improvement despite a reduction in the liver weight to body weight ratio (p=0.006). All hematological and biochemical parameters of UDCA-treated mice were not statistically different from those of *fech/fech* untreated mice (Fig. 2) except TBil which was higher in UDCA-treated mice (p=0.003).

fech/fech mice treated with 0.02 % UDCA showed only a lower ALAT level (p=0.042) and a lower liver weight to body weight ratio (p=0.0014) compared to

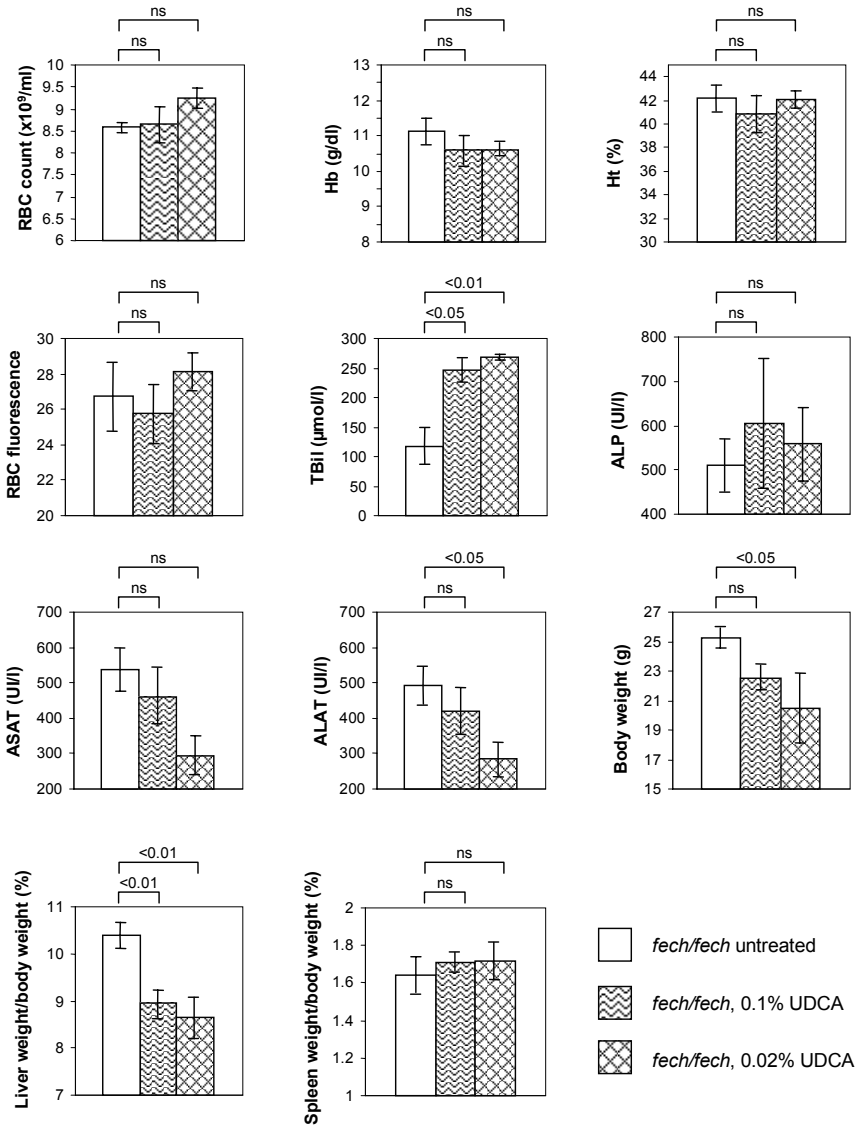

Fig. 2. Biological parameters measured in groups of ten *fech/fech*, five *fech/fech* 0.1 % UDCA-treated and five *fech/fech* 0.02 % UDCA-treated 3-month-old mice. Data shown are mean ± sem. On the top of each graph are shown the p-values of the comparisons between untreated and either 0.1 % or 0.02 % UDCA-treated *fech/fech* mice.

fech/fech untreated mice. All other biological parameters were unaffected by UDCA administration.

Discussion

EPP is one of the inherited disorders originally considered to be transmitted in a Mendelian fashion. However, additional genetic factors must be involved in order to explain phenotypic diversity. The major complication of EPP remains the development of severe hepatobiliary dysfunction progressing to liver failure which is fatal in the absence of liver transplantation. Although less than 2 % of patients are likely to develop this complication, their early identification would greatly help in ensuring appropriate follow-up and care. Recently, Onaga and collaborators identified a genetic risk factor for hepatobiliary complications in a single Japanese family with EPP. In this family, a proband who developed fatal liver failure was found to carry a mutant inactive FECH allele and a low-expressed normal allele, the expression of which was further reduced by CpG methylation of the promoter (Onaga *et al.* 2004). This is the first family in which a genetic risk factor (FECH gene promoter hypermethylation) may indicate the progression of EPP and thus aid in determining the prognosis. However, in the absence of reliable genetic risk factors or biochemical markers to identify patients susceptible to develop the hepatobiliary complications, the development of effective treatments is crucial.

In the human acute hepatic porphyrias,

intravenous administration of hemin inhibits the induction of ubiquitous delta-aminolevulinic acid synthase, reduces the formation of potentially harmful metabolites of porphyrin synthesis and corrects heme deficiency. In erythropoietic protoporphyria, bone marrow is the main site of protoporphyrin production and heme does not inhibit erythroid-specific delta-aminolevulinic acid synthase (Kappas *et al.* 1995). However, heme administration has been useful in treating patients with protoporphyria who develop liver disease (Bloomer *et al.* 1982, Potter *et al.* 1996). HA has also been used to help achieve and maintain remission of hepatic allograft (Dellon *et al.* 2002). In experimental animals, 50 % to 70 % of heme is taken up by the liver (Snyder and Schmid 1965). In theory, the remaining heme may be taken up by bone marrow cells (Potter *et al.* 1996). However, rapid improvement in liver biochemical markers observed in EPP patients treated with hemin suggests that heme therapy enhances hepatic function, probably, by reconstituting hepatic cytochromes and other hepatic hemoproteins (Potter *et al.* 1996). In our EPP mouse model, heme therapy did not improve liver function. TBil and hepatobiliary enzymes (ALP, ASAT, ALAT) were higher in HA-treated mice than in *fech/fech* untreated mice suggesting a toxic effect of HA in these mice. As it was injected intraperitoneally, it is possible that HA was not taken up by the bone marrow and was directly degraded by spleen macrophages and the liver. This would be consistent with the high spleen weight to body weight ratio observed in HA-treated mice. The mice received the same dose of HA than that used in human, which might have been too high. Further experiments with lower doses and/or other routes of administration are necessary to better understand the real effects of heme injections in *fech/fech* mice.

Recent work on *fech/fech* mice has focused on liver pathology, which is a key feature of this model (Libbrecht *et al.* 2003). Meerman and collaborators have shown that bile formation is strongly altered in *fech/fech* mice (Meerman *et al.* 1999). The effect of bile salts on hepatobiliary function is not simple. In humans, chenodesoxycholate and UDCA seem to improve the condition of protoporphyric patients (Van Hattum *et al.* 1986, Pirlich *et al.* 2001). In mice their effects remains controversial. Most of the studies have been made on griseofulvin-induced protoporphyric hepatopathy. Griseofulvin induces a progressive increase in liver weight, hepatic protoporphyrin content (Gschnait *et al.* 1975) and cholestasis (Berenson *et al.* 1991) by decreasing ferrochelatase activity (Brady and Lock 1992). This model mimics EPP hepatobiliary dysfunction. The major point is that protoporphyrin overproduction comes from the liver and not from the bone marrow (Nakao *et al.* 1967, Poh-Fitzpatrick *et al.* 1983). Some authors have shown that UDCA treatment may have a cytoprotective and choleretic effect on griseofulvin-induced hepatic injuries (Berenson *et al.* 1991, Choi *et al.* 1991). Others have not observed any effect of UDCA administration in the same mouse model (Irifune *et al.* 1991). In *mdr2* P-Glycoprotein gene knock-out mice, a mouse model with cholestatic liver disease, UDCA improved liver pathology and decreased ductal proliferation and portal inflammation (Van Nieuwkerk *et al.* 1996). In our EPP mouse model, the commonly used dosage of 0.5 % UDCA dose was toxic. UDCA-treated mice became hypofertile and *fech/fech* pups were smaller and more icteric than pups of untreated breeders. Administration of 0.1 % and 0.02 % UDCA did not improve the hematopoietic and hepatic condition of *fech/fech* mice. We only observed moderate liver weight reduction. In conclusion, UDCA administration does not improve the protoporphyric condition in this ferrochelatase deficient EPP mouse model.

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