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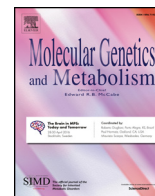
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Delta-aminolevulinic acid synthase 2 expression in combination with iron as modifiers of disease severity in erythropoietic protoporphyria

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A B S T R A C T

Deficiency in ferrochelatase (FECH), the last enzyme in the heme biosynthetic pathway, leads to an accumulation of protoporphyrin IX (PPIX) that causes a severely painful phototoxic reaction of the skin in patients with erythropoietic protoporphyria (EPP). Besides phototoxicity of the skin, EPP patients often present with symptoms of iron deficiency in form of a microcytic and hypochromic anemia with low serum iron and ferritin. In addition, elevated aminolevulinic acid synthase 2 (ALAS2) both at the mRNA and protein levels have been observed among EPP patients. ALAS is the first enzyme in the pathway and exists in two isoforms, whereby the isoform 2 (ALAS2) is expressed exclusively in erythropoiesis. The mRNA of ALAS2 contains an iron response element (IRE) at its 5'UTR. When iron is limited, iron response element binding protein 2 (IRP2) binds to the IRE of ALAS2 mRNA and suppresses its translation. In this study, we demonstrated that iron deprivation increased the amount of ALAS2 mRNA as well as the ratio of ALAS2 to FECH mRNAs in cultured erythroleukemic K562 cells. At the protein level, however, iron deprivation in the cell line caused reductions in both enzymes as shown by the Western blot analysis. A comparable increase in the ratio of ALAS2 to FECH mRNAs was also found in EPP patients indicating an imbalance in heme biosynthesis. As iron cannot be completely missing from an organism, we assume that in EPP patients, a certain amount of ALAS2 mRNA is translated despite a partial deficiency of FECH. The increase in ALAS2 enzyme contributes to the accumulation in PPIX in the patients. Targeted inhibition of ALAS2 could therefore be a treatment option for EPP.

1. Introduction

The heme biosynthetic pathway consists of eight sequential enzymatic steps. The initial and rate-limiting step i.e., the condensation of succinyl-CoA and glycine to form delta-aminolevulinic acid (ALA), is catalyzed by ALA-synthase (ALAS; EC2.3.1.27). ALAS exists in two isoforms, the house-keeping enzyme ALAS1 and the erythroid-specific enzyme ALAS2. In the last step of the pathway, ferrochelatase (FECH; EC4.99.1.1) inserts a ferrous iron (Fe^{2+}) into protoporphyrin IX (PPIX) to give rise to the final product heme. The heme synthesis in the liver is tightly regulated via a negative feedback mechanism on ALAS1 so that intermediates of the pathway are kept at minimal concentrations since they are all toxic to the cells.

A partial deficiency of FECH leads to erythropoietic protoporphyria (EPP; OMIM#177000), a hereditary disorder that affects approximately one in 150'000 inhabitants. The cause of EPP in the majority of the patients are loss-of-function mutations in the *FECH* gene together with a splice modulating single nucleotide polymorphism (SNP) c.315-48 T > C (previously named IVS3-48 t/c [1]); *in trans*. Both c.315-48 T and C alleles produce an aberrantly spliced FECH mRNA that contains a 63 bp long intron 3 sequence. Since there is a premature termination

codon (PTC) present in the intronic sequence, the aberrant splice product is subjected to nonsense mediated decay (NMD). The amount of aberrantly spliced mRNA produced from the C-allele is higher than that from the T-allele. EPP patients with c.315-48C *in trans* to a *FECH* mutation exhibit residual enzyme activities below a threshold of approximately 35% of normal. Very few patients have two separate mutations in the *FECH* gene [2].

As the result of FECH deficiency, PPIX accumulates in erythrocytes and in the endothelial cells of blood vessels. The accumulated PPIX absorbs energy of visible light and releases oxygen radicals [3]. The main symptom of EPP, which usually starts in early childhood, is therefore a severely painful phototoxic reaction of the skin after exposure to the sun and to artificial lights. PPIX is also toxic to liver cells and can thereby cause severe liver damage in 2–5% of the patients. In addition, many patients show signs of iron deficiency in form of a microcytic and hypochromic anemia i.e., low hemoglobin, low ferritin and transferrin saturation and decrease in hepcidine concentration [4,5].

In 2008, a second form of protoporphyria, X-linked erythropoietic protoporphyria (XLEPP; OMIM#300752) was described [6]. The underlying cause of XLEPP are gain-of-function mutations in the *ALAS2* gene, which lead to accumulations of both PPIX and zinc-

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protoporphyrin (ZnPP) in the cells. The cutaneous symptom of XLEPP is indistinguishable from that of EPP. However, the two forms of protoporphyrias seem to differ in the iron metabolism. In XLEPP, iron substitution increases hemoglobin concentration and decreases concentrations of both PPIX and ZnPP. As reported by Landefeld et al., iron substitution was even able to reverse the liver damage in XLEPP patients [7]. As FECH is not affected in XLEPP, by increasing the amount of iron, the second substrate of FECH, excess PPIX can be converted into heme. This could be one explanation for the therapeutic effect of iron on XLEPP.

In EPP however, the effect of iron substitution seems to be ambivalent. Whilst the concentration of hemoglobin increases moderately under iron therapy, growing evidences showed that iron substitution can also increase PPIX concentration [8,9]. The moderate increase in hemoglobin under iron substitution in FECH deficient EPP suggests that iron but not FECH, is the limiting factor in the heme synthesis of EPP [10]. To understand the mechanism behind the increase in PPIX concentration under iron substitution, we previously analyzed blood samples of a number of EPP patients. The result showed that ALAS2 mRNA and, to a lesser extent, ALAS2 protein were increased in these patients [10].

It is known that the mRNA of ALAS2 contains an iron response element (IRE) in the 5'-untranslated region (5'-UTR) [11,12]. When iron is limited, iron response element-binding protein 2 (IRP2) binds to the IRE of ALAS2 mRNA [13]. IREs have been identified in the mRNAs (either in 3'- or in 5'-UTRs) of a number of proteins, mostly related to iron metabolism [14]. And iron response protein exists in two forms, IRP 1 and IRP2. Depending on the cell type and developmental stage, IRP1 and IRP2, either alone or in combination, are present. When iron is abundant, IRP1 binds iron in an iron-sulfur-cluster (4FeS) and acts as cytosolic aconitase while IRP2 is degraded by ubiquitination [15]. In contrast, when iron is limited, both IRPs bind to IREs. Binding of IRPs to IREs in the 5'-UTR such as in ALAS2, suppresses translation of mRNAs, whereas binding of IRPs to IREs in the 3'-UTR stabilizes mRNAs against degradation and therefore enhances translation.

With regards to the pathophysiology of EPP, much focus has so far been placed on the accumulated PPIX due to FECH deficiency. The above-mentioned clinical observations on the adverse effect of iron, the second substrate of FECH, and our finding on the increased expression of ALAS2 bring both these elements into play. In this work, we conducted additional in vitro experiments using an erythroleukemic cell line K562 to study the influence of iron on ALAS2 and FECH at both the mRNA and protein levels. In addition, we expanded the analysis of samples from one of our previous studies that demonstrated a massive increase in ALAS2 expression in relation to FECH expression among EPP patients. The result indicated that not only the FECH deficiency, but also an imbalance in ALAS2 expression contributes to protoporphyrin IX accumulation in FECH deficient EPP.

2. Material and methods

2.1. Cell culture

The human erythroleukemic K562 cell line (American Type Culture Collection) was cultured in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, California, USA) supplemented with 10% fetal calf serum (Bioconcepts Amimed, Allschwil, Switzerland) and streptomycin/penicillin (Gibco, Invitrogen) at 37 °C under 5% CO₂. The cells were split 1:5 every third day to maintain an exponential growth phase. For each experiment, 100,000 cells were washed with medium without antibiotics and seeded in a 6-well cluster plate containing 6 ml fresh medium. The cells were treated with different concentrations of iron chelators desferrioxamine (DFO; at 0, 50, 100, 200 µM) or deferiprone (at 0, 150, 300, 600 µM), with or without the presence of ferric ammonium acetate (at 0, 50, 100, 200 µM) for 24 h or 60 h. Since one molecule of DFO, but three molecules of deferiprone bind one molecule

of iron, the concentration of deferiprone were 3-times higher than that of DFO. All substances were dissolved in autoclaved deionized water and sterile filtered. Within each experiment, the treatments were conducted in duplicates. The K562 cells were collected by centrifugation at the end of treatment.

2.2. RNA isolation and RT-PCR

Total RNA was isolated from cultured K562 cells by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol including the optional on-column DNase digestion step. The quality of RNA was evaluated using RNA Nano chips on an Agilent 2001 bioanalyzer (Agilent Technologies, Santa Clara, California, USA). RNA was then quantified on a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). One-thousand nanograms of total RNA from each isolation were reverse transcribed into cDNA by using the Transcriptor First Strand cDNA Synthesis Kit and anchored-oligo(dT)18 primer (Roche Diagnostics Inc., Basel, Switzerland). For quantification of cDNA, real-time PCR was performed on an ABI PRISM 7000 using gene specific primers and TaqMan probes (ALAS2, Hs01085694_m1; FECH, Hs01555261_m1 and beta-actin, Hs99999903_m1; Applied Biosystems, Life Technologies, Carlsbad, California, USA). One-twentieth of the resulting cDNA was used in each reaction. All measurements were done in triplicates. To obtain the relative concentration of either FECH or ALAS2, the threshold cycle (Ct) value of these transcripts was subtracted by that of β-actin (ΔCt). The concentration was expressed in arbitrary units (a.u.) as $2^{-\Delta Ct} \times 10^3$.

2.3. Western blot analysis

The K562 cells were lysed 60 h after treatment in 100 µl RIPA buffer (ThermoFisher Scientific) supplemented with protease inhibitor (cComplete, Roche). The lysates were incubated for 30 min on ice prior to brief needle sonication (25% amplitude, 6 s pulse) and followed by centrifugation for 15 min at 12,000 g. The supernatants were collected and protein concentration was assessed with the Pierce BCA protein assay kit (ThermoFisher Scientific). Eight micrograms of protein from each sample was denatured in Laemmli buffer (BioRad) containing 1 µl β-mercaptoethanol and loaded onto 10-well 4–20% pre-cast TGX gels (BioRad) for electrophoresis. The transfer of protein onto PVDF membranes (Roche) was carried out for 90 min at 25 W in a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad). Membranes were blocked for 3 h with 3% BSA in 0.05% PBS-T and were incubated with the primary antibody overnight at 4 °C. Membranes were washed three times with 0.05% PBS-T, followed by incubation with the secondary antibody for 1 h in 5% milk and 0.05% PBS-T. After washing with 0.05% PBS-T, signal was detected with the ECL Prime reagent (GE Life Sciences) in a ChemiDoc XRS+ station (BioRad). Chemiluminescence was quantified using the ImageJ software. Ratios were calculated with Excel. Graphical output and statistical analysis were generated with GraphPad Prism software. The following primary antibodies used in the experiment: siFECH (mouse, SantaCruz, sc-377377, 1/500 dilution), siALAS2 (rabbit, Abcam, ab184964, 1/1000 dilution), GADPH (Proteintech, 60004-1-Ig) and corresponding secondary antibodies: anti-mouse (goat, Seracare, 5220-0341, 1/10,000 dilution) and anti-rabbit (goat, Seracare, 5220-0336, 1/10,000 dilution).

2.4. EPP patients and control subjects from a previous study

In one of our previous studies, we published results of quantification of ALAS2 mRNA in peripheral blood samples of 15 EPP patients and 20 control subjects [10]. During that study, we also measured mRNA of the FECH gene, but the results have not been published so far. The diagnosis of EPP in the patients was based on clinical symptoms and a significantly increased protoporphyrin in the erythrocytes. In 12 of the

15 patients, a mutation was identified in the *FECH* gene.

2.5. Statistical analysis

Data from the mRNA experiments in cell culture were analyzed by the Kruskal-Wallis test using the software Analyze-it for Microsoft Excel, version 4.51. Data from patients and control subjects were analyzed by ANOVA using the same software. A $p \leq .05$ was considered to be statistically significant.

3. Results

3.1. Effects of iron availability on *ALAS2* and *FECH* mRNAs in K562 cells

Incubation of K562 cells with iron chelator DFO at different concentrations showed a dose-dependent increase in *ALAS2* mRNA concentration. The effect of DFO was visible after 24 h but was more pronounced after 60 h of incubation. Four separate experiments with a 60-h incubation period were conducted. The results of all four experiments, each in duplicates, were combined ($n = 8$) and showed that the amount of *ALAS2* mRNA increased from 28.8 ± 11.3 a.u. (mean \pm SD) in cells with no DFO added to 38.5 ± 19.9 , 91.3 ± 43.7 and 136.8 ± 61.3 a.u. in cells treated with 50 μ M, 100 μ M and 200 μ M DFO, respectively ($p < .0001$). With respect to the *FECH* mRNA, a slight but not significant variation was observed i.e., 20.0 ± 6.1 , 17.2 ± 7.8 , 14.2 ± 6.3 and 16.1 ± 0.3 a.u. under 0, 50, 100 and 200 μ M DFO, respectively ($p = .3436$). The fold changes in the mRNAs of *ALAS2* and *FECH* after DFO treatment are shown separately in Fig. 1A. We also calculated the ratio between *ALAS2* and *FECH* mRNAs

for each of the eight datasets from all four conditions. As shown in Fig. 1B, the average ratio (mean of eight) changed from 1.5 in cells with no DFO added to 8.4 in cells treated with 200 μ M DFO ($p < .0001$).

To test whether iron supplementation has any influence on mRNA expression, ferric ammonium acetate at concentrations of 0, 50, 100 and 200 μ M were added to the cell culture and incubated for 60 h in two separate experiments. The results ($n = 4$) showed no differences in the amounts of both *ALAS2* ($p = .5533$) and *FECH* ($p = .0851$) mRNAs under these conditions.

To test if a combination of iron and its chelator has any effect on mRNA expression, equimolar amounts of ferric ammonium acetate and DFO (0, 50, 100 and 200 μ M for each substance) were added together to the cells and incubated for 60 h. The result from two separate experiments ($n = 4$) showed no changes in *ALAS2* mRNA ($p = .9363$), but a slight dose-independent variation in *FECH* mRNA under these conditions ($p = .0473$).

The effect of iron chelation on mRNA expression was verified by using another chelator, deferiprone. In one experiment, deferiprone (at 0, 150, 300, 600 μ M) was given to the cell culture and incubated for 60 h. The mRNA concentration of *ALAS2* ($n = 2$) was massively increased in a dose-dependent manner (67.1 ± 7.0 , 184.4 ± 2.2 , 536.0 ± 7.7 and 620.3 ± 5.6 a.u. at 0, 150, 300 and 600 μ M deferiprone, respectively) although this change did not reach a statistical significance with the Kruskal-Wallis test, $p = .0833$. Like DFO, deferiprone did not change *FECH* mRNA significantly under the conditions used in the experiment ($n = 2$; $p = .1038$).

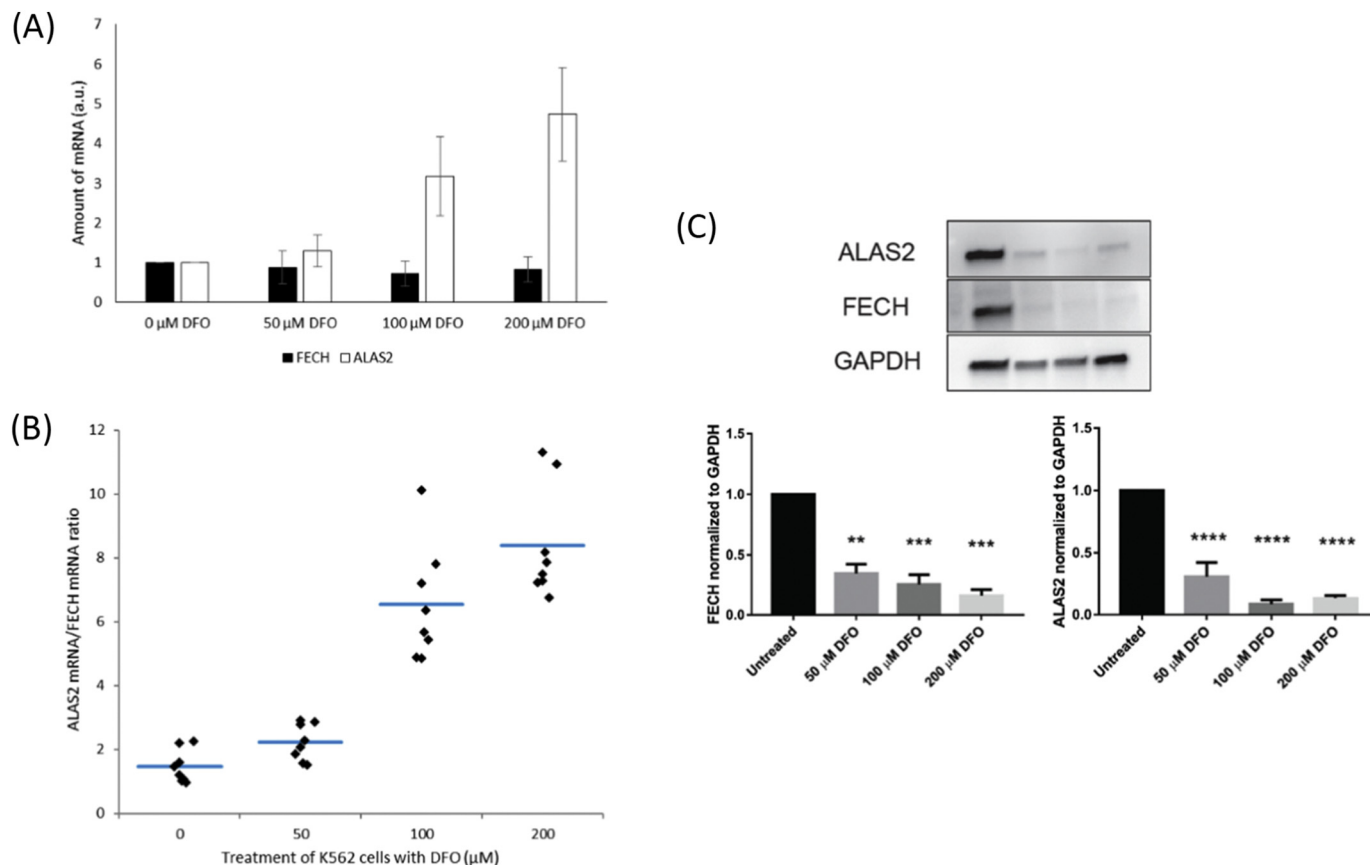


Fig. 1. Changes in *ALAS2* and *FECH* mRNA and protein levels after treatment of K562 cells with different concentrations of deferoxamine (DFO) for 60 h. (A) The results of quantification of *ALAS2* and *FECH* mRNAs in arbitrary units (a.u.) under 0, 50, 100 and 200 μ M of DFO ($n = 8$); (B) The ratio between *ALAS2* and *FECH* mRNAs calculated from the data in Fig. 1A; (C) Western blot analysis of *ALAS2* and *FECH* proteins under the DFO treatment. The amounts of *ALAS2* ($n = 3$; $p = .0001$ ****) and *FECH* ($n = 2$; $p = .001$ at 50 μ M**, $p = .0006$ at 100 μ M*** and $p = .0004$ at 200 μ M***) were quantified after normalizations to GAPDH.

3.2. Effects of iron availability on ALAS2 and FECH proteins in K562 cells

The effect of DFO treatment (0, 50, 100 and 200 μ M) on ALAS2 and FECH proteins was analyzed by Western blot (Fig. 1C). As experimental readouts, ratios were calculated separately for ALAS2 and FECH after normalization to GAPDH under each treatment condition. The readouts from DFO treated cells were then compared to the untreated cells (defined as 1). As shown in Fig. 1C, DFO treatment resulted in sharp decreases in both ALAS2 ($n = 3$) and FECH ($n = 2$).

3.3. Analysis of ALAS2 and FECH mRNAs in EPP patients and control subjects

In 2014 we published a result of quantification of ALAS2 mRNA in peripheral venous blood samples from 15 EPP patients (685.3 ± 313.2 a.u.) and from 20 healthy controls (230.6 ± 191.6), which showed a significant difference between the two groups ($p < .0001$). FECH mRNA measured at that time showed that on average, EPP patients (12.8 ± 12.8 a.u.) had a lower amount of mRNA comparing to controls (22.7 ± 18.1 a.u.). However, the difference did not reach a statistical significance ($p = .0811$) partly due to the relatively large variation in the mRNA of EPP patients ($SD/mean = 0.99$). The top diagram of Fig. 2 displays the result of mRNA quantifications in patients and controls. Like in the cell culture experiments, we calculated the ratio between ALAS2 and FECH mRNAs for each of the 15 patients and 20 control subjects (the bottom diagram of Fig. 2). The difference in the ALAS2/FECH mRNA ratio between these two groups was also statistically significant ($p < .0001$). Ten of the 15 EPP patients carried either nonsense mutations, deletions or insertions in the FECH gene. These mutations produce truncated mRNAs that will be degraded by the nonsense mediated decay (NMD) mechanism. The ALAS2/FECH mRNA ratios from these ten patients were also significantly different from that of the control subjects ($p < .0001$; data not shown). Two of the 15 patients carried missense mutations. Mutations in the remaining three patients were not characterized.

4. Discussion

In this study, we demonstrated that iron deprivation induced by iron chelator DFO led to a dose-dependent increase in ALAS2 mRNA in erythroleukemic K562 cells (approx. 5-fold at the highest DFO dose tested). The ratio between ALAS2 and FECH mRNAs was also increased in a dose-dependent manner, as the treatment of K562 cells with DFO did not significantly change the (absolute) amount of FECH mRNA. In 2013, we conducted a similar experiment and showed that the percentage of correctly spliced mRNA in total FECH transcripts was decreased in the K562 cells treated with DFO, which we could reproduce in the current study (data not shown; [9]). The amount of FECH protein was indeed decreased as shown by the Western blot analysis in both the previous and current studies [9]. As expected, a dose-dependent decrease of ALAS2 protein was observed in the cell culture, presumably as the result of IRP-mediated translational repression of ALAS2 under iron deprivation.

In 2015, we found that FECH-deficient EPP patients had an increase in ALAS2 mRNA comparing to healthy controls [10]. In the present study, we showed that among the same group of EPP patients, the ratio between ALAS2 and FECH mRNAs is also significantly increased compared to the controls. With respect to the FECH mRNA, the mean value from EPP patients was lower than that from the controls. However, the difference was not statistically significant. As mentioned in the Introduction, EPP patients have $\sim 35\%$ of residual FECH enzyme activity. Our measurement of FECH mRNA showed a large variation, especially among the patients. It is not clear at this point whether the variation was due to different mutations that these patients carried or other unknown factors, which could lead to different levels of steady-state mRNA, although most of these patients did carry a null-allele mutation

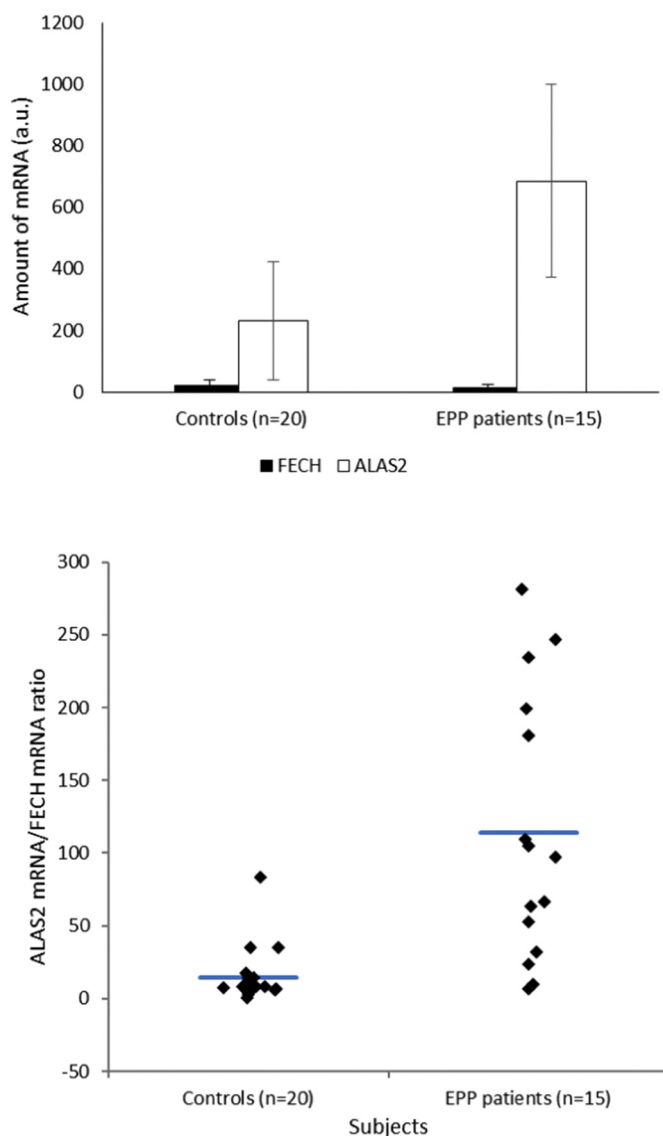


Fig. 2. Quantification of ALAS and FECH mRNA in the peripheral blood samples from EPP patients ($n = 15$) and healthy controls ($n = 20$). The absolute amounts of ALAS2 and FECH mRNA in arbitrary units (a.u.) are displayed in the top diagram. The ratios between ALAS2 and FECH mRNAs from each individual of the patient and control groups are shown in the bottom diagram.

that should induce NMD.

Based on the evidences collected so far, we propose the following model to explain the interaction among iron, ALAS2 and FECH (Fig. 3). In untreated erythroleukemic K562 cells, concentrations of both iron and PPIX, the substrates of FECH, have to be in a fine balance. When iron concentration decreases, for example by treating the cells with DFO, the production of PPIX also needs to be reduced in order to maintain the balance. This is achieved through a translational repression of ALAS2, the rate-limiting enzyme of heme synthesis. At low iron concentration, IRP2 binds to IRE in the 5'UTR of ALAS2 mRNA and suppresses its translation into protein. We assume that the regulation observed in vitro also applies to the situation in vivo. Otherwise, all patients with an iron deficiency anemia would accumulate PPIX and consequently develop signs of EPP.

In this work, we confirmed a finding from our previous study, i.e. iron depletion caused a decrease in FECH protein in the K562 cell line [9]. As expected, we also found a decrease in ALAS2 protein. The IRP mediated translational repression of ALAS2 mRNA has been demonstrated in iron deficiency in various studies [14,16]. In our model, the

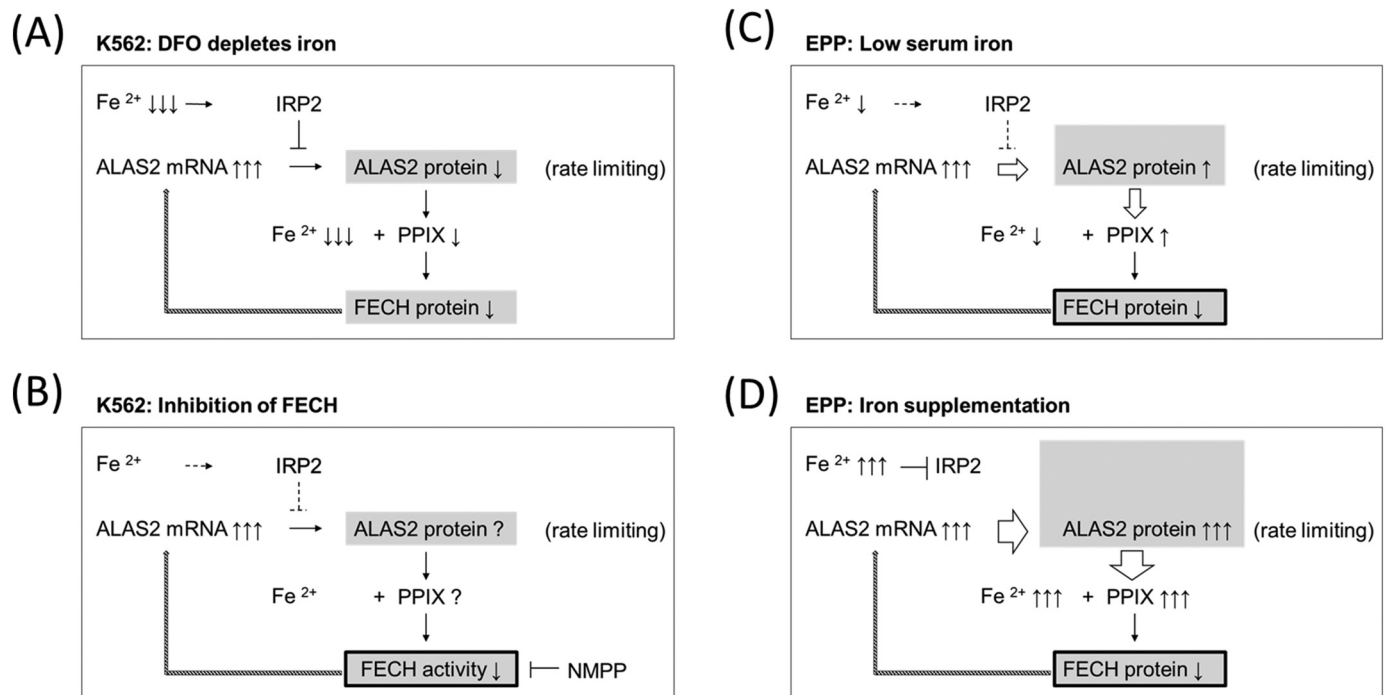


Fig. 3. Interactions among iron, ALAS2 and FECH based on evidences from both cell culture experiments and analyses of blood samples of EPP patients.

decreased iron availability and PPIX production are in line with the reductions in both FECH and ALAS2. The reduced FECH would then lead to an increase in the transcription of ALAS2 mRNA via a yet undefined feedback mechanism. As long as iron is kept at a very low concentration, the increase in mRNA will not result in an increase in ALAS2 protein because of the IRP mediated translational repression (Fig. 3A). In a previous work, we demonstrated that incubation of K562 cells with *N*-methyl protoporphyrin (NMPP), an inhibitor of FECH led to an approximately 2-fold increase in ALAS2 mRNA but no change in the amount of FECH mRNA [10]. This experiment confirmed that a lower FECH activity was sufficient to increase the transcription of ALAS2 mRNA (Fig. 3B).

In EPP, ALAS2 mRNA was increased both in absolute terms and in relation to FECH mRNA as shown in this study. In addition, serum iron concentrations of EPP patients are mostly below the normal range. However, a certain amount of iron is indispensable for life. We therefore hypothesize that iron which is still present, allows translation of a certain amount of ALAS2 mRNA and subsequently causes an increase in the PPIX production (10; Fig. 3C). However, when serum iron concentration increases as in the case with iron supplementation, the IRP mediated translational repression of ALAS2 will be further lifted as IRP2 will be degraded. This will result in a massive increase in ALAS2 protein and in turn, an aggravation of PPIX overproduction since FECH is limited in EPP patients (10; Fig. 3D).

In summary, iron depletion in K562 cells may reduce PPIX synthesis, similar to that of iron deficiency anemia. In EPP, FECH deficiency leads to an increase in ALAS2 mRNA via a yet undefined feedback mechanism. Under low serum iron conditions, a certain amount of the elevated ALAS2 mRNA will be translated into protein leading to PPIX production. The overproduction of PPIX can be further aggravated by iron supplementation. ALAS2 mRNA could therefore be a potential therapeutic target for treatment of EPP. An analogous situation can be found in acute porphyrias, in that the isozyme ALAS1 in the liver is overly active during the metabolic crises (acute attacks). Currently, a clinical trial on a siRNA specifically targeted at ALAS1 in the liver has

shown encouraging results.

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