PHARMACOKINETICS AND DISPOSITION

Modification of CYP2E1 and CYP3A4 activities in haemoglobin E-beta thalassemia patients

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Abstract

Objective Thalassemia disease is a genetic haemoglobinopathy usually associated with an iron overload and some degree of organ impairment. The impact of the disease on the drug metabolising enzyme cytochrome P450 (CYP) is not known. CYP2E1 and CYP3A4 are responsible for the metabolism of a large number of drugs and changes in their activities may have clinical consequences.

Methods Haemoglobin E-β thalassemia paediatric, blood transfusion-dependent patients apparently without complications ($n=35$) and healthy controls ($n=42$) were recruited in this study. The ratios of plasma 6-hydroxychlorzoxazone to chlorzoxazone, and urinary 6-beta-hydroxycortisol (6β-OHF) to cortisol were used as indices for CYP2E1 and CYP3A4 activities, respectively. Blood and plasma samples were assayed for parameters of clinical biochemistry, oxidants and antioxidants.

Results There were significant increases in serum iron, protein carbonyl and lipid peroxidation in thalassemia patients, whereas there was a decrease in blood glutathione, but unchanged plasma nitric oxide metabolites. CYP2E1 activity in the patients was unchanged; however, when the patients were stratified by splenectomy status, CYP2E1 activity was increased in non-splenectomised patients in comparison with the controls and splenectomised subjects. On the other hand, 6β-OHF/cortisol ratios increased markedly in patients associated with depressed growth hormone levels. There were no correlations between CYP2E1 activity and oxidant stress or antioxidant parameters.

Conclusions This report is the first demonstration that thalassemia major is associated with an alteration of CYP2E1 and CYP3A4 activities; this could modify the sensitivity of thalassemia patients to the toxic or therapeutic effects of drugs.

Keywords Thalassemia · CYP2E1 · CYP3A4 ·
Oxidative stress · Hypersplenism · Growth hormone

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Introduction

The hepatic drug-metabolising cytochrome P450 (CYP) enzymes are responsible for the detoxification and metabolic activation of the majority of clinically used drugs and many chemical toxicants. There is substantial inter-individual variation in the activity of CYP enzymes in humans, which can result in decreased or increased susceptibility to the beneficial or adverse effects of drugs. The activity of CYP enzymes including CYP1A2, CYP2E1 and CYP3A4 has been reportedly depressed in the presence of the liver diseases [1, 2]. CYP3A4—the most abundant CYP enzyme in human liver—is involved in the metabolism of more than 50% of currently used drugs. Although the hepatic content of CYP2E1 is much less than that of CYP3A4, the activity and expression of CYP2E1 has been suggested to be linked to oxidative stress and chemical-induced hepatotox-

icity, including alcoholic liver disease and non-alcoholic steatohepatitis [3, 4].

β-Thalassemia is a genetic disease in which an abnormal beta-globin gene results in decreased or absent production of the normal beta globin chain [5]. When β-thalassemia is inherited together with a haemoglobin E (HbE) allele, the resulting condition, HbE-β thalassemia (HbE-β Thal), is sometimes characterised by a severe, transfusion-dependent thalassemia major [5]. A decrease in, or absence of, normal globin chain synthesis results in ineffective erythropoiesis, and an excess of unpaired globin chain in the red blood cells is responsible for autooxidation of proteins and lipid peroxidation of membranes, which leads to haemolytic anaemia [6] The release of iron by haemolysis and the continuing requirement for blood transfusion in thalassemia major inevitably leads to a state of iron overload and the ensuing generation of reactive oxygen species [7].

Iron overload has been demonstrated to be associated with many complications found in thalassemia major, including heart disease and endocrine dysfunction such as growth hormone deficiency, hypogonadism and liver fibrosis [8–10]. Endocrine complications are the most frequent and early complications in thalassemia, especially in patients in developing countries [10]. Deficiency of growth hormone in children has recently been shown to involve CYP3A activity [11, 12]. Consistent with this is the observation that hypophysectomized rats exhibit a large increase in CYP3A expression [13]. In addition, spleen enlargement and a hyperdynamic state of the circulatory system are very commonly found in HbE-β Thal patients [5], due primarily to chronic anaemia compensation. Splenectomy is an established therapeutic intervention in these patients. Hyperdynamic state in spleen-intact patients may affect liver blood perfusion [14, 15], which may eventually change the hepatic drug metabolising enzyme activity. To date, there have been no reports concerning the effect of thalassemia disease on drug metabolising enzymes. In this study, we assessed CYP enzyme activities in paediatric thalassemia patients, because this population is less likely to be afflicted by the overt liver diseases usually found in adult patients [9, 16] that could confound the study.

The present study was aimed at CYP2E1 and CYP3A4, as the activities of these two enzymes may be affected in thalassemia patients, and altered activity may have clinical significance. The study used chlorzoxazone, a widely accepted metabolic test probe for assessment of CYP2E1 activity [17]. Many test probes are currently available for CYP3A4, such as midazolam, triazolam and nifedipine; however, either they require intravenous administration or they introduce certain risks of adverse drug events, and thus they are unsuitable for the vulnerable population in this study. The ratio of endogenous 6β-hydroxycortisol (6β-OHF) to cortisol in urine has been suggested as a useful CYP3A4 probe [18], and the cortisol metabolic ratio has indeed shown a good response to enzyme inducers or inhibitors of CYP3A4 [19].

Methods

Materials

NADPH, glucose-6-phosphate (G6P), glucose-6-phosphate-dehydrogenase (G6PD) grade II from yeast, nitrate reductase from *Aspergillus* species were obtained from Boehringer (Mannheim, Germany). Reduced glutathione, 5,5 dithio-bis-2-nitrobenzoic acid (DTNB), phenacetin, β-glucuronidase, cortisol, 6β-hydroxycortisol, chlorzoxazone (CZX), 6-hydroxychlorzoxazone (6OH-CZX), guanidine hydrochloride, dinitrophenylhydrazine (DNPH), glutathione reductase from baker's yeast, were obtained from Sigma (St. Louis, MO). Meta-phosphoric acid (MPA), 1-methyl-2 vinyl-pyridinum trifate (M2VP) were obtained from Fluka (Buchs, Switzerland.). Other chemicals were of analytical grade.

Subjects

Forty two healthy subjects and 35 thalassemia patients participated in the study. Healthy school pupils (19 male and 23 female) with an average age of 10.2 ± 1.5 years (mean±SD) were carefully screened by physical examination, routine liver function tests, kidney function tests, haemoglobin typing and haematological examinations. They were non-anaemic and had no abnormal haemoglobin. None of them currently took medication. The thalassemia group was paediatric patients of the Department of Paediatrics, Srinagarind Hospital, Khon Kaen University, who have been diagnosed as HbE-β Thal and previously characterised for beta globin gene mutation. All patients received regular blood transfusion approximately every 3–4 weeks to maintain pretransfusion haemoglobin levels of 9 g/dl, and all have been under iron chelation therapy with deferrioxamine. Of the 35 subjects, 18 were splenectomised (10 male and 8 female) and 17 were non-splenectomised (7 male and 10 female) with average ages of 12.6 ± 1 and 11.4 ± 0.9 years, respectively. All patients had no signs of liver cirrhosis or cardiac complications upon physical examination and were apparently healthy and without any concurrent illnesses. Medication in the patients was reviewed and none was known to affect CYP2E1 and CYP3A4 activities. All subjects and their parents were given an explanation regarding the proposed study and written informed consent was obtained prior to the study. The study protocol was approved by the Ethics Review Committee of Khon Kaen University.

Study protocol

All subjects were fasted overnight. Morning urine samples (8.00–9.00 A.M.) were collected for cortisol, 6β-hydroxy-cortisol and hydroperoxide assays. For the study of CYP2E1 activity, chlorzoxazone (250 mg) was powdered and suspended in syrup before being administered orally. Fasting was maintained for an additional 2 h. Venous blood samples (approximately 5 ml) were drawn before and exactly 2 h after drug administration, and aliquots were analysed for complete blood count, liver function and iron studies. Samples were stored at −80°C for subsequent analyses. Samples from the predose period served as controls and were used for determinations of oxidant and antioxidant parameters. Some serum samples were also analysed for growth hormone levels by radioimmunoassay using an Active Growth Hormone IRMA assay kit (Diagnostic System Laboratories, Webster, TX).

Oxidants and antioxidants assays

Assay of malondialdehyde (MDA) was performed in plasma as thiobarbituric acid reactive products (TBAR) [20]. Accumulation of nitrate and nitrite, the end-products of nitric oxide (NO) metabolism used as an index of NO synthase activity, was measured in plasma samples. Nitrate and nitrite were assayed according to a previous described method [21] with modifications. Plasma samples were deproteinised by ultrafiltration using centrifugal concentrators with a molecular weight cut-off of 10 kDa (Nanosep, Pall Filtron, East Hills, NY). The nitrate in the samples was reduced to nitrite by nitrate reductase, and the mixture was then reacted with the Griess reagent. The absorbance of the samples was measured in a microplate reader at a filter wavelength of 540 nm. Protein oxidation, assessed as the protein carbonyl, was assayed by a previously described method [22]. In brief, diluted plasma was treated with 15 mM DNPH in 3.6 M HCl for 1 h in the dark. Protein was precipitated, washed, then dissolved in 6 M guanidine and an absorbance reading taken at 360 nm. The carbonyl content was determined from the absorbance of the DNPH-treated sample subtracted from that of the corresponding non-treated sample, using a molar extinction coefficient of $22{,}000 \ M^{-1} \ cm^{-1}$. Concentration of urinary hydroperoxides was determined by the ferrous ion oxidation xylenol orange (FOX) method [23].

Glutathione (GSH) and glutathione disulphide (GSSG) were assayed using M2VP as a glutathione scavenger. A 100 μl sample of whole blood was reacted with 10 μl 33 mM M2VP (in water) or distilled water and samples were stored for subsequent analysis. Samples were treated with 5% metaphosphoric acid to precipitate protein, and supernatants were used to assay for glutathione according to a previously described enzymatic method [24, 25].

Assay of cortisol and 6β-hydroxycortisol

Cortisol and its metabolite, 6β-hydroxycortisol (6β-OHF) in urine were determined by high performance liquid chromatography (HPLC) based on a previously described method [18]. Briefly, 1 ml urine was added to 1 g Na_2SO_4 containing phenacetin (2 μg /ml) as an internal standard. The mixture was extracted by extraction solvent (ethyl acetate: diethyl ether 40:60). The organic phase was washed with 1 M NaOH saturated with Na_2SO_4 and evaporated to dryness. The residue was reconstituted in mobile phase and injected into an HPLC column (YMC Pack-pro C_{18}, 5 μm, 4.6×50 mm; YMC, Kyoto, Japan). Separation was achieved by gradient elution with eluent A, consisting of 50 mM KH_2PO_4, and acetic acid (99.8:0.2 v/v), and eluent B, consisting of 100 % acetonitrile, using the following gradient profile: $t=0$–10 min, 85 % eluent A; 10–13 min, 85%–80% eluent A; 15–20 min, 80%–73% eluent A; 25–27 min, 73%–50% eluent A; 30–35 min, 50%–85 % eluent A. Retention times were 11.5 min for 6β-OHF, 30.4 min for phenacetin, and 40.0 min for cortisol. Eluent was monitored with a UV detector set at 245 nm. Analytical precision was evaluated by intra- and inter-day assay validation at four different concentrations of 6β-OHF (35–400 ng/ml) and cortisol (15–150 ng/ml). The coefficient of variation values of the intra-day assay and inter-day assay were less than 9% and 10%, respectively for 6β-OHF and less than 5% and 7%, respectively for cortisol. The detection limit of 6β-OHF and cortisol was less than 10 ng/ml.

Assay of chlorzoxazone and metabolite

CZX and its metabolite 6OH-CZX in plasma were analysed by HPLC as previously described [26, 27] with modifications. Briefly, 200 μl plasma samples were incubated overnight at 37°C with β-glucuronidase (1,500 U/ml) and phenacetin (60.3 ng/ml) as an internal standard. Protein was precipitated with 0.72 M perchloric acid and the supernatant was extracted with ethylacetate. The organic phase was evaporated and dissolved in a mobile phase consisting of 0.1 M ammonium acetate, acetonitrile and tetrahydrofurane (73:22:5 v/v). A 20 μl aliquot was injected into an HPLC column (Luna C_{18}, 5 μm, 150×4.6 mm, Phenomenex, Torrance, CA) and eluent was monitored with a UV detector set at 287 nm. Retention times were 7.41 min for 6OH-CZX, 10.8 min for phenacetin, and 20.3 min for CZX. The coefficient of variation values of the intra-day assay and inter-day assay were less than 5% and 6%, respectively, for CZX and less than 7% and 9%, respectively, for 6OH-CZX.

Statistical analysis

Results were expressed as mean±SD. Comparisons between control and patient groups with or without splenectomy were

performed using an analysis of variance and post-hoc test with Duncan's multiple comparison procedures. The comparisons between two groups were done using Student's t-test or Mann-Whitney rank-sum test as appropriate. Association between metabolic ratios of 6OH-CZX/CZX and 6β-OHF/cortisol with oxidant or physiological parameters was analysed by Pearson's correlation. Stepwise multiple regression was used to identify significant determinants for predicting ratios of 6OH-CZX/CZX and 6β-OHF/cortisol. P values less than 0.05 were considered significant.

Results

Blood chemistry

The results of haematological and blood chemistry assays in control and thalassemia groups are shown in Table 1. The haemoglobin levels of patients were lower than those of controls and the serum iron profile clearly showed evidence of chronic iron overload, as serum ferritin, serum iron, transferrin saturation (%) were increased and total iron binding capacity (TIBC) decreased. The total bilirubin, aspartate amino transferase (AST), and uric acid levels were increased in thalassemia subjects ($P<0.05$), whereas alanine amino transferase (ALT) levels were unchanged ($P=0.26$).

Oxidant stress in thalassemia

The oxidant status of the patients was quantified by measurements of blood GSH, lipid peroxidation, protein oxidation and NO formation in plasma and urinary hydroperoxides. Blood GSH was dramatically decreased, whereas protein carbonyl, MDA and urinary hydroperoxides were increased when compared with the control group, as shown in Table 2. However, levels of NO metabolites in the plasma were remained unchanged.

Table 1 Haematological and blood chemistry data of subjects

	Control ($n=42$)	Thalassemia ($n=35$)
Hb (g/dl)	13.0±0.57	7.8±1.06*
Serum ferritin (ng/ml)	56.9±23.6	2111±1490*
Serum iron (μg/ml)	75.4±32.2	161.0±35.1*
TIBC (μg/dl)	312.4±42.9	183.0±51.4*
Transferrin saturation (%)	24.3±9.9	81.4±8.4*
Total bilirubin (mg/dl)	0.44±0.16	1.95±0.58*
ALT (U/l)	14.6±4.9	19.5±24.6
AST (U/l)	26.0±4.7	39.1±27.2*
Uric acid (mg/dl)	4.1±0.76	5.1±1.28*

*Significant difference from controls, $P<0.05$
Values represent mean±SD
Hb Haemoglobin, *TIBC* total iron binding capacity, *ALT* alanine amino transferase, *AST* aspartate amino transferase

Table 2 Oxidative and antioxidant status of controls and HbE-β Thal subjects

	Controls ($n=45$)	Thalassemia patients ($n=35$)
Whole blood GSSG (μM)	15.9±3.8	8.9±9.6*
Whole blood GSH (μM)	756±194	266±132*
Plasma protein carbonyl (nmol/mg protein)	0.72±0.3	0.95±0.23*
Plasma MDA (μM)	2.78±1.24	4.00±1.26*
Plasma nitrate + nitrite (μM)	37.6±8.7	35.3±14.4
Total urinary hydroperoxides (μM)	13.2±6.5	16.3±4.9*

*Significant difference from control group ($P<0.05$)
Values were represent mean±SD
GSH Glutathione, *GSSG* glutathione disulphide, *MDA* malondialdehyde

Since splenectomy in thalassemia patients may be implicated in disease status, analyses of haematological and biochemical data were performed according to splenectomised status. There were no significant differences between splenectomised and non-splenectomised patients regarding haemoglobin, uric acid, ALT and bilirubin levels, whereas there was a slight increase in AST levels ($P<0.05$). Parameters of iron profile, including serum ferritin, serum iron and TIBC, were significantly elevated in splenectomised subjects when compared with non-splenectomised subjects: (mean±SD) 2551±1,207 and 1646±1,650 ng/ml, 185±27 and 142±29 μg/ml, and 216±25 and 158±52 μg/dl, respectively, $P<0.05$. However, there was no differences between the two groups of patients in all oxidant parameters, including blood GSH, MDA, protein carbonyl, NO metabolites and urinary hydroperoxides.

CYP2E1 metabolic ratio

Plots of chlorzoxazone metabolic ratios—an indicator of CYP2E1 activity—are shown in Fig. 1. The 6OH-CZX/CZX ratios varied about 8–10 fold in both patient and control groups. The metabolic ratio of patients and control groups was not significantly different: mean (95% confidence interval: 95% CI), 0.42 (0.35–0.49) and 0.40 (0.34–0.46), respectively, $P>0.05$. The association between the 6OH-CZX/CZX ratio and other relevant parameters was explored. Multiple regression analysis was performed to identify determinants of CYP2E1 activity and it was apparent that splenectomy status was the most significant predictor ($P<0.01$). The patient group was then stratified by splectomy status. The non-splenectomised patients had significantly higher 6OH-CZX/CZX ratios compared with splenectomised patients [mean (95%CI): 0.51 (0.40–0.62) and 0.33 (0.25–0.39), respectively] and controls (Fig. 1) ($P<0.05$).

Fig. 1 Scatter plots of plasma 6-hydroxychlorzoxazone/chlorzoxazone (6OH-CZX/CZX) ratios between controls (*n*=42) and thalassemia subjects (*n*= 35), stratified as splenectomised (*n*= 18) and non-splenectomised patients (*n*=17). *Vertical bars* 25th, 50th (median) and 75th percentiles. * Significant difference from non-splenectomised patients, *P*<0.001
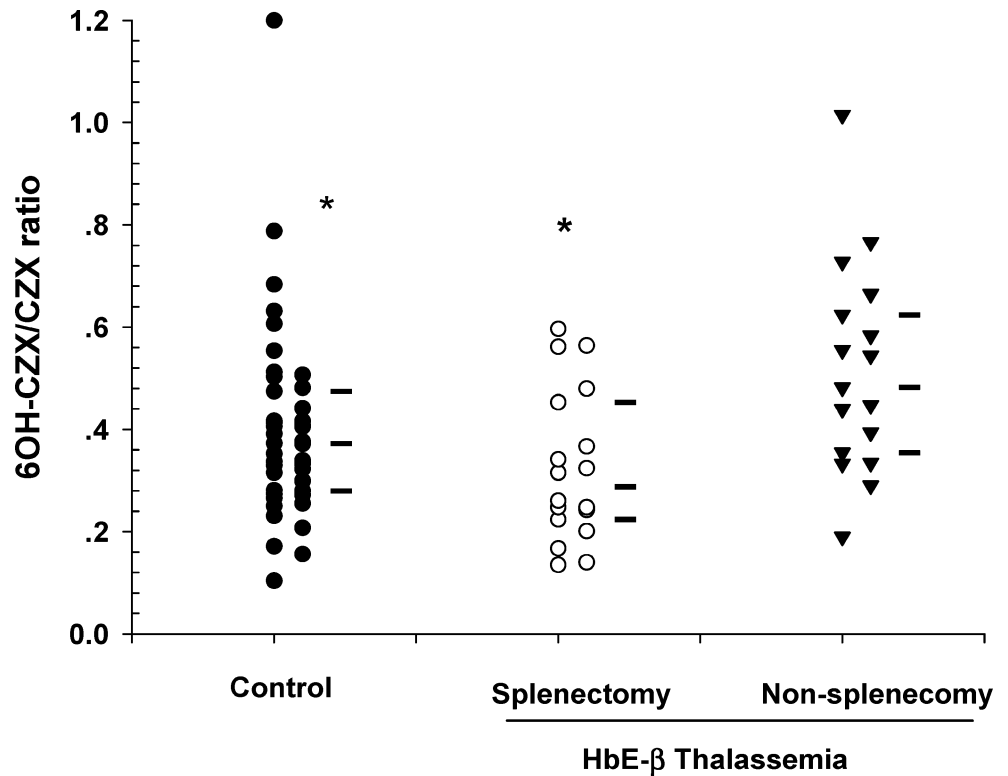
Cortisol metabolic ratio

The urinary 6β-OHF/cortisol ratio was used as an index of CYP3A4 activity. The ratios varied from around 10-fold to 16-fold for patients and controls, respectively. Thalassemia patients showed a higher cortisol metabolic ratio than the control group (Fig. 2) regardless of splenectomy status: mean (95%CI): 5.13 (4.24–6.02) and 2.27 (1.80–2.75), respectively, *P*<0.05. Splenectomised patients had a slightly lower cortisol ratio than non-splenectomised subjects:

mean (95%CI): 4.50 (3.29–5.71) and 5.81 (2.64–7.16), respectively, *P*>0.05.

The 6β-OHF/cortisol ratio was well correlated with oxidant and serum iron parameters, including blood GSH, TIBC, transferrin saturation, haemoglobin and bilirubin, with correlation coefficients of −0.55, −0.61, 0.48, −0.58 and 0.52, respectively, *P*<0.05. The relationship between cortisol ratio and TIBC is showed in Fig. 3. However, when analyses were performed only in the patient group, the correlation became not significant (Fig. 3, inset) except for transferrin saturation, which showed a weak correlation r=−0.38, *P*<0.05. As growth hormone deficiency may be associated with expression of CYP3A4 [12], some samples were assayed for growth hormone levels. Growth hormone levels in thalassemia patients (males=8 and females=8) were significantly lower than in controls (males=6, females=6), mean (95% CI), 0.80 (0.12–1.48) and 2.98 (0.90–5.06), respectively, *P*<0.05.
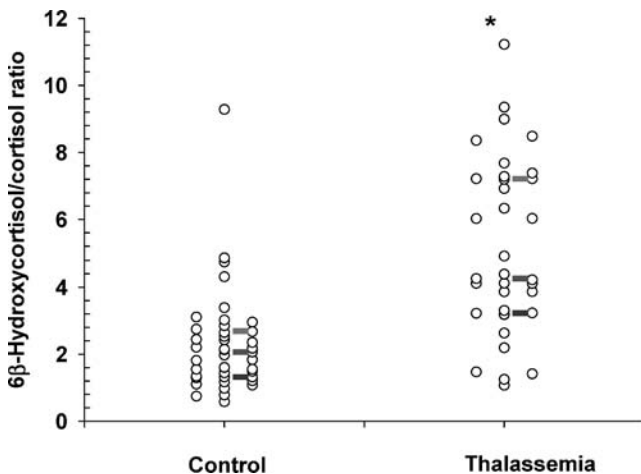
Discussion

The effects of thalassemia disease on the drug-metabolising enzyme cytochrome P450 have not been previously reported. To the best of our knowledge, this report is the first demonstration to show that the activities of CYP2E1 and CYP3A4 are altered in thalassemia disease. Our study was carried out in transfusion-dependent paediatric HbE-β Thal patients who had apparently no signs of complica-

Fig. 2 Scatter plots of urinary 6β-hydroxycortisol (6β-OHF)/cortisol ratios between controls (*n*=42) and thalassemia subjects (*n*=35). *Vertical bars* 25th, 50th (median) and 75th percentiles. * Significant difference from control group, *P*<0.001

Fig. 3 Scatter plot of correlation between total iron binding capacity (TIBC) and urinary 6β-OHF/cortisol ratio in the entire study population: *closed circles* Haemoglobin E-β thalassemia (HbE-β Thal) patients, *open circles* controls. *Inset* A similar plot for the thalassemia population only
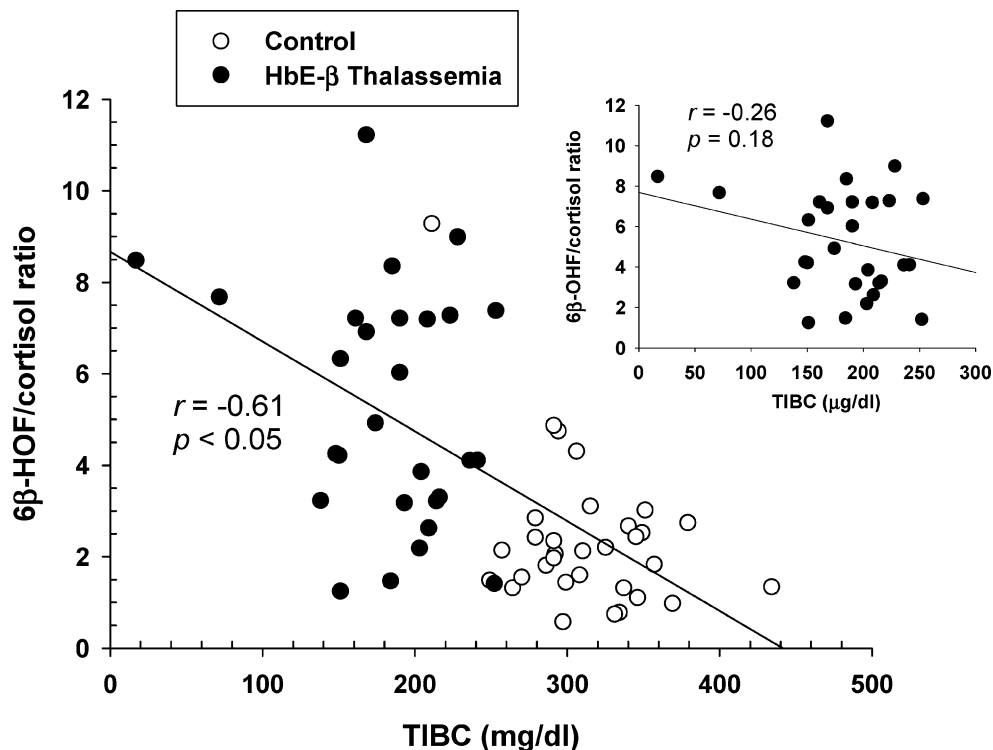
tions. CYP2E1 activity, assessed by chlorzoxazone metabolic ratio, was increased in patients who had not had splenectomy. On the other hand, cortisol metabolic ratios were increased in patients compared with controls. The patients exhibited increased oxidative stress, which is consistent with previous reports [28, 29]. The iron profile of patients indicated an iron overload status. This may be due to suboptimal iron chelation, as most patients in the study were administered desferrioxamine in a relatively low dose. Economic constraints and compliance of (paediatric) patients are the main problems impeding adequate drug doses in these patients.

CYP2E1 has been reportedly down-regulated in oxidative stress conditions induced by ischemic/reperfusion [30], inflammation cytokines [31] and iron overload [32]. Moreover, CYP2E1 is known to be induced by ethanol and other small hydrocarbon molecules as well as by various conditions including diabetes and fasting [33]. There is a high incidence of endocrine disorder including diabetes in thalassemia [8–10]; however, our patients have no symptoms, and laboratory tests indicate no diabetes. In addition, thalassemia patients exhibit iron overload, whereas iron overload has been shown to inhibit the induction of CYP2E1 by ethanol [32]. Therefore, induction of *CYP2E1* or stabilisation of CYP2E1 protein in non-splenectomised patients cannot be explained on the basis of the iron overload or oxidant status.

Chronic anaemia in thalassemia patients causes a hyperdynamic circulation with increased cardiac output and

decreased peripheral resistance [34]. Moreover, thalassemia major is almost always complicated with hypersplenism and, consequently, there is increased splenic and probably portal blood flow [14, 15]. CZX is known to be a high hepatic clearance compound [35], hence its hepatic clearance is significantly increased by an increase in portal blood flow. Hypersplenism is reported to result in a significantly higher splenic and portal blood flow than with a normal-sized spleen [36]. Consequently, there is a greater reduction in portal blood flow with previous hypersplenism than in normal-sized spleens following splenectomy [36]. Therefore, it is inferred that splenectomy may affect thalassemia patients with hypersplenism while not necessarily affecting non-thalassemia with non-splenomegaly patients. This view is consistent with the results of our study, which showed that splenectomy has a negligible effect on cortisol metabolic ratio, as cortisol is a low hepatic clearance compound [37]. Currently, there are no reports on the effect of hypersplenism on drug pharmacokinetics. Since the change in CYP2E1 ratio is relative small, any impact on clinical drug use and chemical exposure remains to be investigated.

A marked increase in the cortisol metabolic ratio is inversely correlated with body iron profile (TIBC and transferrin saturation) and oxidant status (blood GSH). However, this correlation was no longer apparent if analyses were performed in the patient group only (Fig. 3 inset). This suggests that the observed association between cortisol ratio and GSH or iron parameters does not

represent a simple relationship between CYP3A4 activity and redox status or iron profile in thalassemia. CYP3A4 is known to be inducible by various chemicals, for instance anticonvulsants, rifampicin, and ritonavir, as well as endogenous compounds including bile acids and cortisol [38]. However, none of our patients were currently exposed to these compounds. In contrast, plasma cortisol in thalassemia is known to be lower than in healthy individuals [10]. In searching for an explanation of how CYP3A4 activity is up-regulated in thalassemia disease, it can be noted that growth hormone is recognised to be an important determinant of the regulation of CYP3A [39]. Hypophysectomised rats showed marked increases in CYP3A protein and mRNA expression, whereas replacement with growth hormone, but not thyroid hormone, normalised CYP3A levels [13]. Human hepatocyte cultures treated with growth hormone pulses showed suppression of CYP3A4 mRNA and protein levels [40]. A small suppression of CYP1A2 and CYP2D6, but not CYP2E1, was also observed after growth hormone treatment [40]. In a recent study, children with growth hormone deficiency showed a significant increase in urinary 6β-OHF/cortisol ratios in both sexes [12]. Thalassemia disease has been reportedly implicated in endocrine dysfunction, and growth hormone deficiency has a high prevalence (>50%) in children with thalassemia [10]. Our results are consistent with this latter report in that HbE-β Thal patients have significantly lower levels of growth hormone than matched control children. It is suggested that the deficiency in growth hormone is likely to be responsible for the elevation of CYP3A4 activity. Although there is some evidence for differential sexual regulation of rat or human CYP3A isoforms [12, 40], we observed no difference in 6β-OHF/cortisol ratios between male and female patients. Since there was no correlation between growth hormone levels and 6β-OHF/cortisol ratios ($r=−0.26$, $P>0.18$), growth hormone may play a permissive role in the regulation of CYP3A4.

The 6β-OHF/cortisol ratio has been used extensively to evaluate induction or depression of CYP3A4 [19, 41]. Nevertheless, some limitations in the use of this ratio for assessing the CYP3A4 activity have become apparent. It has been recognised that many CYP3A4 probes do not yield consistent results and that they are not strictly correlated to each other [42]. Further study is needed to verify the induction of CYP3A4 activity in thalassemia patients and to investigate the mechanisms underlying the induction. The increased CYP3A4 activity would cause increased rate of metabolism, enhanced oral first-pass metabolism and reduced oral bioavailability, with a resulting decrease in plasma drug concentration. Patients may be at increased risk of therapeutic failure. This may have an impact on drugs used in thalassemia patients, particularly many cardiovascular agents and immunosuppressive agents, which are important drugs used in this group of patients. It is concluded that HbE-β Thal patients have an altered CYP2E1 activity, particularly in non-splenectomised subjects, whereas CYP3A4 activity is increased, probably due to growth hormone insufficiency. The modification of these CYP enzymes may be of clinical significance.

References

1. Tanaka E (1998) Clinical importance of non-genetic and genetic cytochrome P450 function tests in liver disease. J Clin Pharm Ther 23:161–170
2. George J, Byth K, Farrell GC (1996) Influence of clinicopathological variables on CYP protein expression in human liver. J Gastroenterol Hepatol 11:33–39
3. Cederbaum AI (2003) Iron and CYP2E1-dependent oxidative stress and toxicity. Alcohol 30:115–120
4. Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ (2002) Mechanisms of hepatotoxicity. Toxicol Sci 65:166–176
5. Weatherall DJ (2000) Introduction to the problem of hemoglobin E-beta thalassemia. J Pediatr Hematol Oncol 22:551
6. Shinar E, Rachmilewitz EA (1990) Oxidative denaturation of red blood cells in thalassemia. Semin Hematol 27:70–82
7. Weatherall DJ (1998) Pathophysiology of thalassaemia. Baillieres Clin Haematol 11:127–146
8. Borgna-Pignatti C, Rugolotto S, De Stefano P, Zhao H, Cappellini MD, Del Vecchio GC, Romeo MA, Forni GL, Gamberini MR, Ghilardi R, Piga A, Cnaan A (2004) Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. Haematologica 89:1187–1193
9. Cunningham MJ, Macklin EA, Neufeld EJ, Cohen AR (2004) Complications of beta-thalassemia major in North America. Blood 104:34–39
10. Gulati R, Bhatia V, Agarwal SS (2000) Early onset of endocrine abnormalities in beta-thalassemia major in a developing country. J Pediatr Endocrinol Metab 13:651–656
11. Levitsky LL, Schoeller DA, Lambert GH, Edidin DV (1989) Effect of growth hormone therapy in growth hormone-deficient children on cytochrome P-450-dependent 3-N-demethylation of caffeine as measured by the caffeine 13CO2 breath test. Dev Pharmacol Ther 12:90–95
12. Sinues B, Mayayo E, Fanlo A, Mayayo E Jr, Bernal ML, Bocos P, Bello E, Labarta JI, Ferrandez-Longas A (2004) Effects of growth hormone deficiency and rhGH replacement therapy on the 6beta-hydroxycortisol/free cortisol ratio, a marker of CYP3A activity, in growth hormone-deficient children. Eur J Clin Pharmacol 60:559–564
13. Oinonen T, Lindros KO (1995) Hormonal regulation of the zonated expression of cytochrome P-450 3A in rat liver. Biochem J 309:55–61
14. Aessopos A, Farmakis D, Tsironi M, Deftereos S, Tassiopoulos S, Konstantopoulos K, Rombos J, Papalambros E (2004) Hemodynamic assessment of splenomegaly in beta-thalassemia patients undergoing splenectomy. Ann Hematol 83:775–778
15. Garnett ES, Goddard BA, Markby D, Webber CE (1969) The spleen as an arteriovenous shunt. Lancet 1:386–388

16. Li CK, Chik KW, Lam CW, To KF, Yu SC, Lee V, Shing MM, Cheung AY, Yuen PM (2002) Liver disease in transfusion dependent thalassaemia major. Arch Dis Child 86:344–347

17. Frye RF, Stiff DD (1996) Determination of chlorzoxazone and 6-hydroxychlorzoxazone in human plasma and urine by high-performance liquid chromatography. J Chromatogr B Biomed Appl 686:291–296

18. Homma M, Beckerman K, Hayashi S, Jayewardene AL, Oka K, Gambertoglio JG, Aweeka FT (2000) Liquid chromatographic determination of urinary 6beta-hydroxycortisol to assess cytochrome p-450 3A activity in HIV positive pregnant women. J Pharm Biomed Anal 23:629–635

19. Galteau MM, Shamsa F (2003) Urinary 6beta-hydroxycortisol: a validated test for evaluating drug induction or drug inhibition mediated through CYP3A in humans and in animals. Eur J Clin Pharmacol 59:713–733

20. Draper HH, Squires EJ, Mahmoodi H, Wu J, Agarwal S, Hadley M (1993) A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. Free Radic Biol Med 15:353–363

21. Verdon CP, Burton BA, Prior RL (1995) Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by NADP+ when the Griess reaction is used to assay for nitrite. Anal Biochem 224:502–508

22. Reznick AZ, Packer L (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol 233:357–363

23. Banerjee D, Jacob J, Kunjamma G, Madhusoodanan UK, Ghosh S (2004) Measurement of urinary hydrogen peroxide by FOX-1 method in conjunction with catalase in diabetes mellitus-a sensitive and specific approach. Clin Chim Acta 350:233–236

24. Liang Q, Carlson EC, Donthi RV, Kralik PM, Shen X, Epstein PN (2002) Overexpression of metallothionein reduces diabetic cardiomyopathy. Diabetes 51:174–181

25. Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 27:502–522

26. Frye RF, Adedoyin A, Mauro K, Matzke GR, Branch RA (1998) Use of chlorzoxazone as an in vivo probe of cytochrome P450 2E1: choice of dose and phenotypic trait measure. J Clin Pharmacol 38:82–89

27. Leclercq IA, Farrell GC, Field J, Bell DR, Gonzalez FJ, Robertson GR (2000) CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. J Clin Invest 105:1067–1075

28. Chakraborty D, Bhattacharyya M (2001) Antioxidant defense status of red blood cells of patients with beta-thalassemia and Ebeta-thalassemia. Clin Chim Acta 305:123–129

29. Kassab-Chekir A, Laradi S, Ferchichi S, Haj Khelil A, Feki M, Amri F, Selmi H, Bejaoui M, Miled A (2003) Oxidant, antioxidant status and metabolic data in patients with beta-thalassemia. Clin Chim Acta 338:79–86

30. Pahan K, Smith BT, Singh AK, Singh I (1997) Cytochrome P-450 2E1 in rat liver peroxisomes: downregulation by ischemia/reperfusion-induced oxidative stress. Free Radic Biol Med 23:963–971

31. Hakkola J, Hu Y, Ingelman-Sundberg M (2003) Mechanisms of down-regulation of CYP2E1 expression by inflammatory cytokines in rat hepatoma cells. J Pharmacol Exp Ther 304:1048–1054

32. Stal P, Johansson I, Ingelman-Sundberg M, Hagen K, Hultcrantz R (1996) Hepatotoxicity induced by iron overload and alcohol. Studies on the role of chelatable iron, cytochrome P450 2E1 and lipid peroxidation. J Hepatol 25:538–546

33. Lieber CS (1997) Cytochrome P-4502E1: its physiological and pathological role. Physiol Rev 77:517–544

34. Aessopos A, Farmakis D, Hatziliami A, Fragodimitri C, Karabatsos F, Joussef J, Mitilineou E, Diamanti-Kandaraki E, Meletis J, Karagiorga M (2004) Cardiac status in well-treated patients with thalassemia major. Eur J Haematol 73:359–366

35. Liangpunsakul S, Kolwankar D, Pinto A, Gorski JC, Hall SD, Chalasani N (2005) Activity of CYP2E1 and CYP3A enzymes in adults with moderate alcohol consumption: a comparison with nonalcoholics. Hepatology 41:1144–1150

36. Takagi K, Ashida H, Utsunomiya J (1994) The effect of splenomegaly on splanchnic hemodynamics in nonalcoholic cirrhosis after distal splenorenal shunt and splenopancreatic disconnection. Hepatology 20:342–348

37. Charmandari E, Johnston A, Brook CG, Hindmarsh PC (2001) Bioavailability of oral hydrocortisone in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. J Endocrinol 169:65–70

38. Burk O, Wojnowski L (2004) Cytochrome P450 3A and their regulation. Naunyn Schmiedebergs Arch Pharmacol 369:105–124

39. Redmond GP, Bell JJ, Nichola PS, Perel JM (1980) Effect of growth hormone on human drug metabolism: time course and substrate specificity. Pediatr Pharmacol (New York) 1:63–70

40. Dhir RN, Dworakowski W, Thangavel C, Shapiro BH (2006) Sexually dimorphic regulation of hepatic isoforms of human cytochrome p450 by growth hormone. J Pharmacol Exp Ther 316:87–94

41. Wenk M, Todesco L, Krahenbuhl S (2004) Effect of St John's wort on the activities of CYP1A2, CYP3A4, CYP2D6, N-acetyltransferase 2, and xantine oxidase in healthy males and females. Br J Clin Pharmacol 57:495–499

42. Watkins PB (1994) Noninvasive tests of CYP3A enzymes. Pharmacogenetics 4:171–184