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## Effects of growth hormone deficiency and rhGH replacement therapy on the 6 $\beta$ -hydroxycortisol/free cortisol ratio, a marker of CYP3A activity, in growth hormone-deficient children

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**Abstract Objective:** To determine the effect of both growth hormone deficiency (GHD) and rhGH replacement therapy on CYP3A activity as well as the potential influence of gender.

**Methods:** The sample consisted of 35 GHD children (16 males and 19 females), aged 2.9–13.1 years, and a control group of 35 healthy children matched for age and sex. The urinary ratio 6 $\beta$ -hydroxycortisol/free cortisol was used as a marker of CYP3A activity. In patients, urine samples were collected at two times, prior to starting rhGH replacement treatment and 30 days after beginning therapy.

**Results:** A significantly higher metabolic activity in GHD children was observed in relation to controls ( $P=0.0001$ ) without sex differences. Paired comparisons demonstrated a sexually dimorphic effect of rhGH therapy on the CYP3A activity. While boys displayed a

significant decrease ( $P=0.003$ ), girls showed no significantly different values of CYP3A marker ( $P>0.05$ ). Unpaired comparison between controls and GHD children after therapy demonstrated absence of significant differences in boys ( $P>0.05$ ) and a strikingly higher activity in girls ( $P=0.0001$ ).

**Conclusions:** The data suggests that: (a) GHD in children increases CYP3A activity in a non-sex-dependent manner, (b) rhGH treatment for 30 days to GHD children results in a sexually dimorphic effect on CYP3A activity, with a significant decrease in males toward normalization in relation to controls and non-significant changes in females. The results of this study may have important clinical implications for GHD children, since changes in CYP3A activity importantly affect the metabolism of both steroid hormones and CYP3A substrate drugs.

**Keywords** rhGH therapy · CYP3A4 activity · GH-deficiency

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### Introduction

CYP3A is the predominant constitutive hepatic cytochrome  $P_{450}$  (CYP) in humans and is responsible for approximately 60% of the total drug oxidations [1]. Hydroxylations of endogenous steroids, such as testosterone and estradiol, are catalyzed by CYP3A [2]. Bioactivation of genotoxicants aflatoxin-B1 [3] and heterocyclic amines [4] is also produced by this  $P_{450}$  isoform. CYP3A activity is characterized by its inter-individual variability, which importantly accounts for the variable pharmacokinetics of its substrates [5]. Changes in activity of this cytochrome, produced by inducers or inhibitors, are known to cause important clinical consequences in terms of either efficacy or toxicity [6, 7].

Growth hormone (GH) is recognized to be the main determinant of the expression and regulation of CYPs in the liver of rodents [8], and the importance of transcription factors related to tissue-specific gene expression has been emphasized [9]. Sex differences in CYP expression in rodents are dependent on the sexual dimorphism in the secretion pattern of GH [10]. While male rats present high concentration peaks in the blood, between which the baseline concentration is almost undetectable, in female rats, the peaks are lower but appear more frequently, and the baseline concentration is higher than in males [11, 12].

Recently, it has been demonstrated that pulsatile or continuous GH exposure decreases or increases, respectively, CYP3A activity [13]. There exists some information about the regulatory properties of GH on CYP enzymes in humans based on a few studies on children [14–17] and adults [18–20]. Despite the fact that sexual dimorphism in GH secretion in humans is well documented [21], gender differences in the effect of GH on CYPs have not been reported so far. In addition, the influence of GH-deficiency status on CYP activity has not been studied in humans.

The aim of the present study is to examine the separate and interactive effects of growth hormone deficiency (GHD) and rhGH replacement therapy on CYP3A activity in children, as well as to investigate the potential gender influence.

## Material and methods

### Subjects and study protocol

The study population consisted of 35 selective GH-deficient children with no other pituitary alteration (16 males and 19 females) aged  $9.5 \pm 3.2$  years, ranging from 2.9 years to 13.1 years. Physical examinations and laboratory tests confirmed that patients had no liver or kidney dysfunction. For the control group, we recruited 35 healthy children matched for age ( $\pm 6$  months) and sex with patients.

All controls were of normal height and weight. We confirmed that the control children were healthy on the basis of physical examination and routine laboratory tests. Both patients and controls were asked to refrain from ingesting any drugs or foods known to alter CYP3A activity 1 month prior to and during the entire study period.

The rhGH therapy for GH-deficient children was approved by the Spanish Committee for GH Treatment, on the basis of a peak GH concentration greater than 5 ng/ml with two tests: insulin and either clonidine or ornitine in children above the age of 5 years or clonidine and ornitine in children below the age of 5 years. One blood sample was obtained from each patient to measure insulin-like growth factor I (IGF-I) and IGF-binding protein 3 (IGFBP-3). Patients received once daily subcutaneous injection of rhGH at 2200 hours, at

a dose of 0.166 mg/kg per week. Children and their parents were adequately informed, and they gave their informed consent to participate in the study, which was approved by the Ethics Committee for Clinical Research of the Hospital “Miguel Servet” of the University of Zaragoza, Spain. Controls and GHD children were white Caucasians from the same geographical area (North of Spain).

Overnight urine samples (interval from 2200 hours to 0800 hours) were collected from patients at two times: day 0 (prior to starting treatment) and day 30 (30 days after beginning rhGH treatment). In the control group, a unique urine sample was obtained, which was collected at the same time interval. Aliquots (15 ml) were immediately frozen at  $-80^{\circ}\text{C}$  until analysis.

### Analytic methods

$6\beta$ -Hydroxycortisol was quantified according to the procedure described by Lee [22].  $6\beta$ -Hydroxycortisone was used as internal standard. Urine extraction was performed through an activated Sep-Pack C18 cartridge washed with 10 ml of 4% methanol. The column was eluted with 10 ml of ethyl acetate. After washing the eluate, it was evaporated to dryness in a  $37^{\circ}\text{C}$  bath under a gentle stream of nitrogen. The residue was dissolved in 0.2 ml of  $\text{CH}_3\text{OH}$ , and an aliquot of 20  $\mu\text{l}$  of the solution was injected into the high-performance liquid chromatography (HPLC) system. The reverse phase column was a  $100 \times 8$  mm Radial-Pack Novapack C18 (Millipore Ibérica S.A.) with a 5- $\mu\text{m}$  particle size (Waters Corporation, Madrid, Spain), which was eluted isocratically with a mobile phase containing 0.01 M  $\text{KH}_2\text{PO}_4$ /acetonitrile/methanol (84/5/11, v/v/v) at a flow rate of 2 ml/min and a pressure of 1500 psi. The compounds were detected by ultraviolet absorbance at 244 nm. The chromatograph was a LC Module I Plus equipped with Millennium 2010 software (Waters Corp., Madrid, Spain). We used a Waters 996 photodiode array detector (Waters Corporation, Madrid, Spain) to compare the spectral of the peaks with the spectral libraries of standards. All reagents were of analytical grade for HPLC.  $6\beta$ -Hydroxycortisol and  $6\beta$ -hydroxycortisone were purchased from Sigma Chemical, Co., Madrid, Spain. Within-run and between-run coefficients of variation were greater than 5.5% and 6.2%, respectively. The recovery ranged from 92% to 95% for three different concentrations of  $6\beta$ -hydroxycortisol, and the limit of quantification was 26 nmol/l. Urinary free cortisol concentrations were assayed using a fluorescence polarization immunoassay method (TDX System, Abbott Diagnostics, Madrid, Spain), following the instructions of the manufacturer, after an extraction of the unconjugated cortisol with methylene chloride. Within- and between-day coefficients of variation were lower than 5%. For data analysis,  $6\beta$ -hydroxycortisol and free cortisol concentrations were expressed as nanomole per liter. The

metabolic ratio was calculated by the quotient  $6\beta$ -hydroxycortisol/free cortisol, as indicator of CYP3A activity. Serum GH, total IGF-I and IGFBP-3 concentrations were measured by immunoradiometric assays by Diagnostic System Laboratories (Webster, Tex., USA).

### Statistical analysis

Non-parametric statistical methods were selected, since the data distributions were neither normal nor fitted to an ordinal scale. We used the Wilcoxon test or the Mann–Whitney test for paired or unpaired comparisons, respectively. The associations between variables were performed using the Spearman correlation test. Data are presented as medians and 95% confidence intervals for a better description. A  $P$  value of  $<0.05$  was considered to be statistically significant. The statistical analysis was done using the SPSS 11.0 software for Windows.

## Results

First, we investigated the influence of the age and gender on the marker of CYP3A activity. Correlation analysis and unpaired comparisons showed lack of influence of the age or sex on the  $6\beta$ -hydroxycortisol/free cortisol ratio ( $P>0.05$ ) both in the control group ( $r=0.07$  and  $Z=-0.9$ ) and the group of GHD children ( $r=0.06$  and  $Z=-1.45$ ). There were no significant associations between the CYP3A marker and either serum GH, IGF-I or IGFBP-3 ( $P>0.05$  in all cases).

Table 1 summarizes unpaired and paired comparisons of the metabolic ratio between groups. GHD children displayed a strikingly higher activity than controls ( $P<0.0001$ ). The differences were also significant in the two subgroups established by gender ( $Z=-2.37$ ,  $P=0.01$  for males, and  $Z=-2.7$ ,  $P=0.007$  for females).

With regard to the effect of rhGH treatment for 30 days (Table 1), the  $6\beta$ -hydroxycortisol/free cortisol

ratio did not change significantly in the total group of children ( $Z=-1.09$ ,  $P>0.05$ ). However, when males and females were analyzed separately, a significant decrease was observed after rhGH treatment in relation to day 0 in males ( $Z=-2.99$ ,  $P<0.02$ ). In contrast, in females, although median values tended to be higher, no significant changes were detected ( $Z=-1.44$ ,  $P>0.05$ ). Baseline serum GH, IGF-I or IGFBP-3 had no influence on the changes of the cortisol metabolic ratio ( $P>0.05$  for the three correlation coefficients).

The results of the unpaired comparisons of the metabolic ratio between GH-treated children (30 days) and controls are illustrated in Table 1. Male children showed a normalization of the CYP3A marker toward values non-statistically different from those of controls ( $Z=-0.07$ ,  $P>0.05$ ). In contrast, females showed the opposite effect with statistically higher values than controls ( $Z=-3.86$ ,  $P<0.001$ ).

## Discussion

To evaluate CYP3A activity, we selected the urinary ratio  $6\beta$ -hydroxycortisol/free cortisol, as it is a non-invasive test. The interval of urine collection from 2200 hours to 0800 hours was chosen for several reasons. First, this time interval is more comfortable than that of 24 h, since in most cases, it implies only the collection of the first urine in the morning. Moreover, subcutaneous administration of rhGH takes place at 2200 hours, and hence the highest GH plasma concentrations are reached at night, mimicking the physiological high nocturnal GH secretion [23]. In addition, a good correlation has been reported between cortisol metabolic ratio in 24-h urine and in overnight urine [24].

The present study shows, to our knowledge, the first observation of a higher CYP3A activity in GH-deficient children in relation to controls (Table 1). The mechanism underlying this effect is unclear. Some observations in rodents demonstrate that GH suppresses CYP3A2 expression in rat liver. CYP3A2 is the

**Table 1** Effect of growth hormone (GH) deficiency and rhGH treatment (30 days) on the CYP3A activity marker. *CI* confidence interval of the median, *GHD* growth hormone deficiency

	Medians and 95% CI			Comparisons ( $P$ value)		
	Controls	GHD children		GHD baseline versus controls	rhGH therapy versus GHD baseline	rhGH therapy versus controls
		Baseline	After rhGH therapy			
Total group ( $n=35$ )						
Median	1.67	2.52	2.30	0.0001	0.27	0.004
95% CI	1.46–1.88	2.1–3.49	1.78–3.54			
Males ( $n=16$ )						
Median	1.78	3.40	1.62	0.01	0.003	0.94
95% CI	1.45–1.93	2.45–5.24	0.99–3.39			
Females ( $n=19$ )						
Median	1.59	2.21	3.33	0.007	0.14	0.0001
95% CI	1.3–1.74	1.85–2.88	2.08–3.54			

steroid and xenobiotic-metabolizing enzyme in the rat that is constitutively expressed in prepubertal male and female rats [25, 26], and it does not require the presence of GH [27]. Moreover, hypophysectomy leads to increased expression of CYP3A2 in rats [28], and, conversely, overexpression of GH in transgenic mice results in both lower CYP3A2 expression and CYP3A2 enzyme activity [29].

Another novel finding of this work is the sexual dimorphism in the effect of rhGH treatment on CYP3A activity (Table 1). We have observed a marked decrease in male children toward normalization in relation to controls. In contrast, in females, the CYP3A values did not change significantly.

Previous studies conducted to examine the effect on drug metabolism of GH treatment given to GHD children show contradictory results without reference to sex differences [14–17]. Redmond et al. [14] found a significant increase in elimination half-life on amobarbital in six children after hGH therapy for 6 weeks. In contrast, these same authors reported a decrease in plasma half-life of theophylline in four children following the same therapeutic scheme. Redmond et al. [15] and Levitski et al. [16] noted a significant decrease in caffeine N-3-demethylation in six GHD children after 1 month on hGH therapy. Recently, Gil-Berglund et al. [17] observed a higher CYP3A activity in 14 GHD children after 3 months on rhGH replacement therapy.

The sexual dimorphism in CYP3A activity found here could be related to the changes in GH exposure profile as a consequence of the daily s.c. administration of rhGH. The GH secretory pattern is sexually different in humans [21] and rodents [12]. Males secrete in episodic bursts with undetectable GH levels between peaks. In contrast, females present more frequent pulses of a lower magnitude than those in males, whereas the interpulse concentrations of GH are always measurable [30, 31]. Recently, in GHD adults, it has been demonstrated that the type of GH pulses is an important factor of GH actions on CYP3A and other metabolic parameters, with an increase in CYP3A activity when GH is administered continuously (mimicking the feminine profile) and a tendency to decrease when pulses mimic the GH masculine profile [13].

The dimorphic sexual effect of GH in rodents on the expression of CYP3A2 and other cytochromes is well documented [28, 31–33], as well as the differential regulation of constitutive CYPs by GH pulse pattern [34]. GH mediates the activation of the transcriptional factor STAT 5b in a manner that enables it to act as a transcriptional activator of hepatic CYP3A2 and other genes in response to male GH pulses [35]. Pulsatile GH, which is a characteristic feature of males, increases STAT 5b activation by tyrosine phosphorylation and nuclear translocation [35, 36]. The low nadir GH concentrations between pulses in males sensitize phosphotyrosine phosphatases, which allow STAT 5b to be newly phosphorylated in the following peak [37]. Continuous GH exposure, similar to the pattern of adult female rats,

leads to desensitization of tyrosine phosphorylation and, consequently, to lower STAT5b activation [38].

Nevertheless, whether or not the regulation of STAT 5b depends on the secretory profile of GH in humans is unknown. However, as suggested by Jaffe et al. [13], a daily subcutaneous injection of rhGH, which has been administered to children in this study, mimics the nearly continuous feminine profile. Therefore, male GH-deficient children treated with rhGH could suffer a relative “feminization” of their GH plasma pattern with a presumable loss of some regulatory actions of GH. This could explain the decreased values of the CYP3A marker in boys from baseline to 30 days after beginning rhGH therapy with a normalization in relation to controls.

In GHD females treated with rhGH, CYP3A activity has shown a marked increase regarding controls (Table 1). A possible explanation could arise from the change in their GH profile toward more elevated and continuous levels of plasma GH. As referred to previously, the continuous profile of GH delivery increases CYP3A activity [13]. This is consistent with the higher CYP3A activity measured by the erythromycin breath test found in acromegaly patients [39], since in acromegaly, GH levels are higher, and the physiological low nadir GH concentrations are never reached [40]. The higher CYP3A activity in women in relation to men found in a previous work [20] could also be due to the more continuous hepatic exposure to GH in women, since a lack of sex differences in CYP3A activity has been found in hepatic microsomes [1]. To further reinforce this argument, the continuous presence of GH in vitro induced CYP3A4 expression in primary hepatic cells [41].

The urinary ratio 6 $\beta$ -hydroxycortisol/free cortisol, in spite of being one of the most successfully used biomarker to indicate CYP3A activity [42–45], also measures that of CYP3A5 isoform. *CYP3A5* is polymorphically expressed, and subjects with the variant allele *CYP3A5\*3* have improper mRNA splicing and, consequently, lower catalytic activity [46]. A possible limitation of the present study is that we did not determine genotypes of *CYP3A5*. Therefore, differences between controls and patients in the proportion of carriers of the allelic variant *CYP3A5\*3* could presumably account for the differences found in the cortisol metabolic ratio. However, the low prevalence of the rapid phenotype/genotype in white Caucasian people (5–10%) [47, 48] makes this possibility remote.

In summary, the data suggests that: (a) GH deficiency in children produces increased CYP3A activity in a non-sex-dependent manner, (b) rhGH treatment for 30 days to GHD children causes a sexually dimorphic effect on CYP3A activity, with a decrease in males towards normalization in relation to controls. These data clearly support the notion of the central role of GH in determining the level of expression of some metabolic genes. Since changes in CYP3A activity importantly affect the metabolism of pharmacological, environmental or endogenous substrates, such as steroid hormones, the

results of this study should be taken into account to avoid potential undesirable consequences in GH-deficient children.

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