Plasma concentrations of fibroblast growth factors 21 and 19 in patients with Cushing's syndrome

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Short title: Fibroblast growth factors 21 and 19 in Cushing's syndrome

SUMMARY

The objective of this study was to measure plasma fibroblast growth factor 21 and 19 (FGF21 and FGF19) levels in patients with Cushing's syndrome (CS) and to compare it with those of lean control subjects (C) and patients with obesity (OB).

14 untreated patients with CS, 19 patients with OB and 36 C were included in the study. Plasma FGF21 and FGF19 levels were measured by ELISA kits, other hormonal and biochemical parameters were measured by standard laboratory methods.

Plasma FGF19 did not significantly differ among the studied groups. Plasma FGF21 levels were significantly higher in both CS and OB groups relative to C group but they did not differ between CS and OB groups. In a combined population of all three groups FGF21 levels positively correlated with BMI, waist circumference and percentage of total and truncal fat mass. Less prominent inverse relationship with these parameters was found for FGF19. Neither FGF21 nor FGF19 were significantly related to cortisol concentrations.

Increased FGF21 concentrations in both patients with CS and OB relative to lean subjects suggest that excessive body fat and/or related metabolic abnormalities rather than direct effects of cortisol are responsible. In contrast neither obesity nor hypercortisolism significantly affected FGF19 concentrations.

Keywords: fibroblast growth factor 21; fibroblast growth factor 19; hypercortisolism; obesity

INTRODUCTION

Members of the fibroblast growth factor (FGF) family are involved in numerous cellular processes including growth, angiogenesis, and development (Böttcher and Niehrs 2005, Grose and Dickson 2005, Presta *et al.* 2005). Recent findings indicate that FGF21 and FGF19 may regulate metabolic homeostasis in paracrine and/or endocrine manner (Tomlinson *et al.* 2002, Fu *et al.* 2004, Kharitonenkov *et al.* 2005, Wente *et al.* 2006).

FGF21 is expressed primarily in liver (Nishimura *et al.* 2000). Recent studies have demonstrated that FGF21 is powerful stimulator of glucose uptake and lipolysis in adipose tissue (Kharitonenkov *et al.* 2005). Experimental studies have shown strong antiinflammatory, antidiabetic and hypolipidemic effects of FGF21 administration in both rodents and primates (Kharitonenkov *et al.* 2005, Wente *et al.* 2006, Kharitonenkov *et al.* 2007). In mice, the expression of FGF21 in liver is tightly nutritionally regulated. It is increased by starvation and ketogenic state (Inagaki *et al.* 2007, Badman *et al.* 2007, Lundåsen *et al.* 2007) and decreased by feeding (Badman *et al.* 2007). FGF21 levels are also regulated by nutritional state in humans. They are increased in patients with obesity and/or type 2 diabetes mellitus and decreased in malnourished patients with anorexia nervosa (**Dostálová *et al.* 2008, Dostálová *et al.* 2009)**.

FGF19 is another recently characterized member of FGF family (Nishimura *et al.* 1999, Xie *et al.* 1999). Transgenic mice overexpressing human FGF19 have increased metabolic rate and decreased adiposity (Tomlinson *et al.* 2002, Fu *et al.* 2004). Circulating FGF19 exhibits a diurnal rhythm controlled by transintestinal bile acid flux. This diurnal rhythm of FGF19 is abolished by fasting (Lundåsen *et al.* 2006). In humans, FGF19 originates from intestine and modulates hepatic bile acids synthesis (Jones 2008). To our knowledge the only information concerning changes of FGF19 in humans is available in our

recently published paper showing that severe malnutrition in patients with anorexia nervosa does not affect circulating levels of FGF19 (Dostálová *et al.* 2008).

To our best knowledge neither FGF21 nor FGF19 have been studied in patients with Cushing's syndrome. Here we tested the hypothesis that chronically increased cortisol levels in patients with Cushing's syndrome may alter circulating levels of FGF21 and FGF19. Such alterations could in turn contribute to some metabolic disturbances seen in these patients. Since patients with Cushing's syndrome frequently suffer from visceral obesity, insulin resistance/diabetes, arterial hypertension and other abnormalities similarly to patients with metabolic syndrome we compared their concentrations of FGF21 and FGF19 with both lean healthy control subjects and patients with obesity and type 2 diabetes mellitus.

METHODS

Study subjects

Fourteen patients with active Cushing's syndrome (12 women, 2 men, age: 45.8 ± 3.39 yrs, body mass index (BMI): 33.7 ± 1.42 kg/m^2), nineteen obese patients (17 women, 2 men, age: 54.6 ± 3.3 yrs, BMI: 45.8 ± 2.42 kg/m^2) and thirty six healthy controls (28 women, 8 men, age: 43 ± 2.07 yrs, BMI: 22.7 ± 0.26 kg/m^2) were included in the study.

The diagnosis of Cushing's syndrome (CS) was based on clinical status, diminished circadian rhythm of cortisolaemia (nocturnal cortisol over 150 nmol/l), unsuppressed cortisolaemia in 1 mg Dexamethasone test (cortisol over 86 nmol/l) and increased free urinary cortisol excretion (urinary free cortisol over 500 nmol/day). All 14 patients with CS had arterial hypertension, 12 of them had been already treated pharmacologically, 11 of them with a combination of antihypertensive drugs (with ACE inhibitors or sartans in 10 cases, calcium

channel blockers in 6 cases, beta-blockers in 8 cases, diuretics in 7 cases, imidazoline receptor antagonist in 1 case and an alpha-blocker in 1 case). All patients had clinical signs of abdominal fat accumulation as measured by increased waist circumference. Twelve patients had disturbances in serum lipid spectrum (10 patients had elevated total and LDL (low density lipoprotein) cholesterol levels, in 7 cases together with elevated triglycerides, in 3 patients also with decreased HDL (high density lipoprotein) cholesterol levels; 2 patients had only elevated triglycerides and decreased HDL cholesterol serum concentrations). One patient had been already treated with a combination of statin and fibrate. One patient was diagnosed with impaired glucose tolerance (IGT), nine patients with type 2 diabetes mellitus (DM) - four of them had been already treated, three with antidiabetic drugs (PAD), one with a combination of PAD and insulin.

The group of patients with obesity (OB) was characterized by BMI over 30 kg/m^2. Sixteen out of nineteen patients in this group had arterial hypertension, all had been already on antihypertensive treatment, thirteen of them on a combination of antihypertensive drugs (with ACE inhibitors or sartans in 14 cases, calcium channel blockers in 4 cases, beta-blockers in 10 cases, diuretics in 11 cases and imidazoline receptor antagonist in 3 cases). Fifteen patients had pathological serum lipid spectrum (10 patients had elevated total and LDL cholesterol levels, in 2 cases together with elevated triglycerides and decreased HDL cholesterol, in 5 cases only with HDL cholesterol decrease; 2 patients had elevated triglycerides in combination with decreased HDL cholesterol and 3 patients had isolated HDL cholesterol decrease). Eight patients had been already treated, either with fibrate or statin, one patient with a combination of both. Two patients had impaired fasting glucose (IFG) levels, four patients were diagnosed with IGT and nine patients had type 2 DM, all treated either with PAD, insulin or its combination.

The healthy controls (C) had no history of obesity or malnutrition, arterial hypertension, glucose or lipid metabolism disturbances, malignant tumors or other severe co-morbidities. They had no regular medication (except for hormonal contraceptives in 5 women). Blood tests confirmed normal blood count, biochemical and hormonal parameters.

All subjects were asked to fast and drink only water a night long prior to the study. Written informed consent was signed by all participants before being enrolled in the study. The study was approved by the Ethical Committee, 1st Faculty of Medicine, Charles University and General Teaching Hospital, Prague, Czech Republic, and was performed in accordance with the guidelines proposed in the Declaration of Helsinki.

Anthropometric examination and blood sampling

All patients were examined in the morning at a basal state. All subjects were weighted and measured.

A percentage of truncal body fat was assessed by body composition measurement using Dual-Energy X-Ray Absorptiometry (DEXA, Hologic Discovery, USA). Percentage of total body fat was examined by bioimpedance (Multi-frequency Bodystat QuadScan 4000, Douglas, British Isles) at body current flow of 5, 50, 100 and 200 kHz, respectively. Resting energy expenditure (REE) and respiratory quotient (RQ) were measured by indirect calorimetry (V Max Encore 29N, Viasys, Pennsylvania, USA) performed with a ventilated hood system. Oxygen consumption and carbon dioxide production were measured, and energy expenditure was calculated by using the Weir formula (Weir 1949). Blood samples for FGF21, FGF19, resistin, adiponectin, leptin, insulin and biochemical parameters measurement were withdrawn between 7 and 8 a.m. after 12 hours of overnight fasting. Plasma samples for FGF21 and FGF19 analysis were collected into polypropylene tubes containing Na_2EDTA.

Plasma was separated by centrifugation at room temperature and stored at - 80 ^{o}C until being assayed.

Hormonal and biochemical assays

Plasma FGF21 concentrations were measured by a commercial ELISA kit (BioVendor, Brno, Czech Republic). The samples were diluted with Dilution Buffer 1:1 or 2:1, respectively. The sensitivity was 5.0 pg/ml, and the intra- and interassay variability was 5.0 and 9.0 %, respectively. Plasma FGF19 concentrations were measured by a commercial ELISA kit (BioVendor, Brno, Czech Republic). The samples were diluted with Dilution Buffer 1:1. The sensitivity was 4.8 pg/ml, and the intra- and interassay variability was 7.0 and 8.5 %, respectively.

Serum insulin concentrations were measured by a commercial RIA kit (Cis Bio International, Gif-sur-Yvette, France). Sensitivity was 2.0 µIU/ml, and the intra- and interassay variability was 4.2 and 8.8 %, respectively. Serum leptin concentrations were measured by a commercial ELISA kit (BioVendor, Brno, Czech Republic). Sensitivity was 0.12 ng/ml, and the intra- and interassay variability was 1.7 and 8.0 %, respectively. Serum adiponectin concentrations were measured by a commercial ELISA kit (Linco Research, St. Charles, Missouri, USA). Sensitivity was 0.78 ng/ml, and the intra- and interassay variability was 3.4 and 5.7 %, respectively. Serum resistin concentrations were measured by a commercial ELISA kit (BioVendor, Brno, Czech Republic). Sensitivity was 0.2 ng/ml, and the intra- and interassay variability was 3.1 and 6.5 %, respectively.

Serum levels of insulin-like growth factor-1 (IGF-1) were measured by a commercial IRMA kit (Immunotech, Prague, Czech Republic). Sensitivity was 2 ng/ml, and the intra- and interassay variability was 6.3 and 6.8 %, respectively. Plasma levels of cortisol were measured

by a commercial RIA kit (Immunotech, Prague, Czech Republic). Sensitivity was 10 nmol/l, and the intra- and interassay variability was 5.8 and 9.2 %, respectively. **Serum biochemical parameters (glucose, total and HDL-cholesterol and triglycerides) were measured by standard laboratory methods on Hitachi analyzer, the value of LDL-cholesterol was calculated. Glycated hemoglobin was analyzed by high performance liquid chromatography (HPLC) on Variant II BioRad analyzer. TSH, fT4 and fT3 were measured using chemiluminiscence imunoassay (CLIA) on ADVIA: Centaur analyzer.**

Statistical analysis

The statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA). Results are expressed as means ± standard error means (SEM) or median, upper and lower quartiles, minimum and maximum values. ANOVA test, followed by Dunn's test was used for groups' comparison. The correlations between the values were estimated by Spearman correlation test. A p value < 0.05 denoted statistical significance.

RESULTS

Anthropometric and metabolic characteristics of study subjects

Ten out of fourteen patients with CS had BMI in the range of obesity, and all had signs of abdominal fat accumulation with waist circumference increased over normal values (>80 cm in women, >94 cm in men). The waist circumference of OB patients was significantly higher in comparison with CS group (Table 1). The systolic and diastolic blood

pressure was significantly higher in both CS and OB groups relative to C, but it was also significantly higher in patients with CS as compared to OB group (Table 1).

The percentage of total and truncal body fat was significantly higher in CS and OB subjects relative to C. Percentage of total body fat was significantly higher in OB relative to CS group. REE per kg was significantly higher in C in comparison with CS and OB with no difference in CS vs. OB, whereas RQ was significantly lower in OB patients relative to CS and C, with no difference between CS and C subjects (Table 1).

Serum levels of hormonal and biochemical parameters: comparison of CS, OB and C subjects

Fasting serum glucose, insulin, HOMA index (homeostatic model assessment of insulin resistance) and glycated hemoglobin were significantly higher in both CS and OB patients relative to C, but with no significant difference between CS and OB group (Table 1). HDL cholesterol was significantly reduced in CS and OB relative to C, but did not differ between CS and OB subjects. Triglycerides were significantly increased in CS and OB group in comparison with C. There were no significant differences in total and LDL cholesterol levels between the studied groups (Table 1).

Both fasting serum leptin and resistin were significantly higher in CS as compared to C. Leptin levels were significantly higher in OB relative to C subjects. No significant differences were found in adiponectin levels between the examined groups (Table 1).

TSH, free T3 and free T4 were significantly lower in CS as compared to OB and C. Basal plasma cortisol was significantly higher in CS relative to OB and C patients. There were no significant differences in serum IGF-1 concentrations between the studied groups (Table 1).

Plasma levels of FGF21 and FGF19: comparison of CS, OB and C subjects

Fasting plasma FGF21 levels were significantly higher in CS and OB subjects relative to C. There was no significant difference in FGF21 levels between CS and OB patients (Figure 1).

Plasma FGF19 levels did not significantly differ between the groups studied. (Table 1). N**o statistically significant gender differences within the groups either in FGF21 or in FGF19 levels were found.**

Relationship of FGF21 and FGF19 with other anthropometric, hormonal and biochemical parameters

The relationship of FGF21 and FGF19 with other studied parameters was calculated in the combined population of all three groups (Table 2).

Plasma FGF21 levels significantly positively correlated with BMI, waist circumference and percentage of total and truncal fat mass, systolic and diastolic blood pressure, triglyceride levels, HOMA index, insulin, glycated hemoglobin, leptin levels and were inversely related to HDL-cholesterol and adiponectin levels (Table 2).

Plasma FGF19 was significantly positively associated with HDL cholesterol levels and correlated inversely with BMI, waist circumference, percentage of total and truncal fat mass, systolic blood pressure, HOMA index, insulin, glycated hemoglobin and IGF-1 levels (Table 2).

We failed to find significant relationships between FGF21 and FGF19 and plasma cortisol, thyroid hormones**,** total and LDL cholesterol or resistin levels, respectively (Table 2).

DISCUSSION

Numerous previous studies have shown that regulation of metabolic homeostasis is a very complex process involving not only traditional central and peripheral mechanisms but also novel factors produced by adipose tissue, liver and muscle (Anderlova *et al.* 2007; Haluzik *et al.* 2009; Havel 2002). FGF21 is an example of such factor being produced predominantly in the liver and to lesser degree in the adipose tissue (Dostálová *et al.* 2009; Kharitonenkov and Shanafelt 2008). Here we show that plasma FGF21 levels are markedly increased in patients with Cushing's syndrome relative to healthy lean control subjects but they do not significantly differ from those of patients with obesity and type 2 diabetes mellitus. This finding indicates that increased FGF21 concentrations in patients with Cushing's syndrome are very likely due to obesity and metabolic abnormalities induced by chronic hypercortisolism rather than due to direct effect of chronic hypercortisolaemia on FGF21 production *per se*. Nevertheless, it should be noted that the cross-sectional design of our study does not allow us to unambiguously distinguish between the direct effects of cortisol and effects of obesity and related metabolic alterations, respectively. On the other hand, while FGF21 concentrations positively correlated with BMI, waist circumference and percentage of total and truncal fat mass, no such relationship was found between FGF21 and circulating cortisol levels arguing rather for an indirect mechanism.

Changes of FGF21 levels in patients with Cushing's syndrome are in agreement with previous findings that the presence of obesity and/or type 2 diabetes mellitus is accompanied by elevated FGF21 concentrations (Zhang *et al.* 2008, **Mraz *et al.* 2008**). We have recently shown that FGF21 mRNA expression is detectable not only in liver but also in both subcutaneous and visceral adipose tissue (**Mraz *et al.* 2008**). While no differences in FGF21 mRNA expression in subcutaneous fat were found between the obese and the lean subjects, FGF21 mRNA expression in visceral fat increased significantly in the obese relative to

the lean group. Nevertheless, adipose tissue mRNA expression was approximately 100-fold lower as compared to liver (*Mraz et al.* 2008).

FGF21 administration in both rodents and primates markedly improved numerous metabolic abnormalities including blood glucose, serum triglyceride and HDL levels (Kharitonenkov *et al.* 2005, Wente *et al.* 2006, Kharitonenkov *et al.* 2007). Furthermore, FGF21 treatment also decreased body weight in primates but not in mice (Kharitonenkov *et al.* 2005, Kharitonenkov *et al.* 2007). Increased FGF21 concentrations in patients with obesity and Cushing's syndrome thus may seem somewhat paradoxical given the fact that these patients display insulin resistance, obesity and dyslipidemia. We suggest that increased FGF21 levels in these patients may represent a compensatory response to improve impaired insulin sensitivity and other unfavorable metabolic features.

In contrast to altered FGF21 concentrations we did not see any major differences in FGF19 levels either in patients with obesity or Cushing's syndrome relative to lean healthy individuals. Experimental studies in transgenic mice overexpressing FGF19 have shown numerous obvious metabolic changes including increased metabolic rate, decreased adiposity, increased food intake, decreased lipids and increased insulin sensitivity (Tomlinson *et al.* 2002, Fu 2004). In contrast to FGF21 no data from primate studies concerning FGF19 administration are available suggesting the possibility of distinct functions of FGF19 in rodents and humans. Our results show that circulating FGF19 is not regulated either by nutritional status, the presence of insulin resistance/diabetes and/or chronically increased cortisol levels. We are aware that our results are based on a single measurement after overnight fasting and possible nutrition-related changes in the dynamic secretion pattern of FGF19 or its local alterations in the site of its production may not have been noticed here.

In conclusion, our study has shown that FGF21 levels are increased in both patients with simple obesity and obese patients with endogenous hypercortisolism while no such alterations were found in FGF19 concentrations. We suggest that increased FGF21 concentrations in patients with Cushing's syndrome are rather due to their excessive fat accumulation and related metabolic abnormalities than due to a direct effect of cortisol on FGF21 production.

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Table 1. Anthropometric, biochemical and hormonal characteristics of patients with Cushing's syndrome (CS), obesity (OB) and control subjects (C).

	CS (n=14)	OB (n=19)	C (n=36)
Age (years)	45.8 ± 3.39	54.6 ± 3.3	43.0 ± 2.07
Body mass index (kg/m^2)	33.7 ± 1.42 *	45.8 ± 2.42 *	22.7 ± 0.26
Waist circumference (cm)	110.6 ± 3.38 *,□	127.2 ± 4.26 *	77.5 ± 1.72
Systolic blood pressure (mmHg)	153.6 ± 5.2 *,□	134.5 ± 3.96 *	111.0 ± 2.92
Diastolic blood pressure (mmHg)	91.4 ± 3.41 *,□	77.5 ± 1.94 *	70.0 ± 1.83
% of total body fat (bioimpedance)	40.0 ± 2.37 *,□	51.1 ± 2.44 *	18.8 ± 2.01
% of truncal body fat (DEXA)	44.0 ± 1.33 *	41.2 ± 2.8 *	20.7 ± 1.19
REE/kg (kcal/day/kg)	17.9 ± 0.42 *	16.0 ± 0.75 *	22.5 ± 0.47
Respiratory Quotient	0.79 ± 0.02 □	0.71 ± 0.01 *	0.79 ± 0.01
Glucose (mmol/l)	6.4 ± 1.03 *	7.8 ± 0.85 *	4.5 ± 0.12
Insulin (µUI/ml)	85.1 ± 38.66 *	44.4 ± 10.57 *	15.3 ± 1.4
HOMA index	20.7 ± 8.01 *	15.3 ± 3.69 *	3.2 ± 0.35
Glycated hemoglobin A1c (%)	5.6 ± 0.6 *	5.7 ± 0.48 *	3.7 ± 0.07
Total cholesterol (mmol/l)	5.7 ± 0.38	4.9 ± 0.21	5.1 ± 0.14
LDL-cholesterol (mmol/l)	3.6 ± 0.32	2.9 ± 0.22	3.1 ± 0.12
HDL-cholesterol (mmol/l)	1.2 ± 0.06 *	1.1 ± 0.07 *	1.5 ± 0.08
Triglycerides (mmol/l)	2.1 ± 0.27 *	2.7 ± 0.89 *	1.0 ± 0.07
Leptin (ng/ml)	60.2 ± 5.36 *	55.7 ± 4.49 *	9.3 ± 1.38
Resistin (ng/ml)	7.5 ± 0.65 *	7.9 ± 1.38	5.4 ± 0.34
Adiponectin (ng/ml)	17.0 ± 1.84	17.8 ± 1.83	22.4 ± 1.75
fT3 (pmol/l)	4.1 ± 0.17 *,□	5.1 ± 0.47	5.1 ± 0.16
fT4 (pmol/l)	13.7 ± 0.67 *,□	16.2 ± 0.51	15.8 ± 0.46
TSH (mIU/l)	0.9 ± 0.19 *,□	2.2 ± 0.33	2.3 ± 0.59
IGF-1 (ug/l)	282.5 ± 33.89	199.1 ± 20.10	223.0 ± 22.12
Basal plasma cortisol (nmol/l)	946.3 ± 8.2 *,□	515.3 ± 38.4	611.4 ± 33.75
FGF19 (pg/ml)	149.8 ± 22.54	172.0 ± 26.96	234.8 ± 26.6

* $p<0.05$ vs. C group

□ $p<0.05$ for CS vs. OB group

Values are means ± SEM. Statistical significance is from one-way ANOVA.

DEXA = Dual-Energy X-Ray Absorptiometry; REE = resting energy expenditure; HOMA = homeostatic model assessment of insulin resistance; LDL = low density lipoprotein; HDL = high density lipoprotein; fT3 = free triiodothyronine; fT4 = free thyroxine; TSH = thyroid stimulating hormone; IGF-1 = insulin-like growth factor-1; FGF19 = fibroblast growth factor 19

Table 2. Relationships of fibroblast growth factor 21 and 19 (FGF21 and 19) with anthropometric, biochemical and hormonal parameters calculated in a combined population of patients with Cushing's syndrome, obesity and lean healthy controls (n = 69).

	FGF21		**FGF19**	
	R	**p**	**r**	**p**
Waist circumference	0.413	0.003	-0.324	0.02
BMI	0.344	0.006	-0.279	0.017
% of total body fat	0.393	0.016	-0.283	0.044
% of truncal body fat	0.428	0.009	-0.334	0.043
sBP	0.367	0.007	-0.271	0.048
dBP	0.439	<0.001	NS	NS
HDL-cholesterol	-0.323	0.01	0.299	0.0143
Triglycerides	0.504	<0.001	NS	NS
Glucose	0.421	<0.001	NS	NS
Insulin	0.498	<0.001	-0.274	0.023
HOMA index	0.544	<0.001	-0.305	0.013
HbA1c (%)	0.351	0.005	-0.285	0.016
Leptin	0.438	<0.001	NS	NS
Adiponectin	-0.261	0.046	NS	NS
IGF-1	NS	NS	-0.325	0.022

r = correlation coefficient; p = statistical significance; NS = non-significant

Results are from Spearman Correlation Test.

BMI = body mass index; sBP, dBP = systolic, diastolic blood pressure; HDL = high density lipoprotein; HOMA = homeostatic model assessment of insulin resistance; HbA1c = glycated hemoglobin; IGF-1 = insulin-like growth factor-1.

Figure legend

Figure 1. Plasma fibroblast growth factor 21 (FGF21) concentrations in patients with Cushing's syndrome (CS), obesity (OB) and control subjects (C). Values are median, upper and lower quartiles, minimum and maximum data. Statistical significance is from one-way ANOVA and Dunn's test.

* $p<0.05$ vs. C group