Brief Genetic Analysis

Corticosteroid Binding Globulin Gene Polymorphism Influences Cortisol Driven Fat Distribution in Obese Women

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Abstract

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Hypothalamo-pituitary-adrenal axis has been reported to influence fat mass distribution in obesity. We investigated the hypothesis that corticosteroid-binding globulin (CBG) polymorphism could influence obesity, metabolic, or hypothalamo-pituitary adrenal (HPA) axis activity parameters. In 44 obese pre-menopausal women, a microsatellite located within the CBG gene was analyzed, providing three genotypes: 86/86 ($n = 29$), 86/90 ($n = 14$), and 90/90 ($n = 1$). No significant difference was found for obesity, metabolic, and HPA axis activity parameters between the genotypes 86/86 and 86/90. Looking for differences in correlations between HPA axis activity parameters and obesity or metabolic parameters between the two genotypes, genotype 86/90 showed a strong correlation between salivary cortisol after dexamethasone (0.25 mg) suppression test and waist-to-hip ratio ($r = -0.84$, $p = 0.0007$), whereas this correlation was weaker for genotype 86/86 ($r = -0.34$, $p = 0.09$). These data were completed with an analysis of the BclI polymorphism of the glucocorticoid receptor (GR) gene. There was an association between this GR polymorphism and both awakening salivary cortisol and postdexamethasone salivary cortisol but no association for obesity or metabolic parameters. We concluded that CBG gene polymorphisms might modulate the influence of the HPA axis on the fat mass distribution in this population.

Key words: glucocorticoid receptor, transcortin, hypothalamo-pituitary-adrenal axis, fat mass distribution

Visceral fat accumulation represents a cardiovascular risk factor independently of total adiposity and leads to the metabolic syndrome (1). Chronic increased cortisol secretion such as in Cushing's syndrome leads to both visceral fat accumulation and the metabolic syndrome. However, the relationship between visceral obesity and non-tumoral hypothalamo-pituitary adrenal (HPA)[1] axis activity remains to be clarified. Several studies have reported moderate hyperactivity of the HPA axis in visceral fat accumulation (2–5). This HPA axis hyperactivity was suggested to be of central origin (2,3) or to be the result of an increased cortisol clearance in obese subjects (6). On the other hand, a peripheral origin with local hypercortisolism has been recently well documented in a report on the role of 11β-hydroxysteroid dehydrogenase Type 1 in adipose tissue (7,8).

Genetic and environmental effects influence fat mass and visceral fat distribution. However, genetic influence is more important for visceral fat accumulation than for total fat mass (9). As to genes regulating obesity through the HPA axis, the glucocorticoid receptor gene stands out as the best replicated candidate gene. The BclI polymorphism within the intron 2 of the glucocorticoid receptor gene has been associated with elevated cortisol concentrations, visceral obesity, hypertension, and insulin resistance (10).

Recently, we reported that corticosteroid-binding globulin (CBG) gene polymorphisms strongly influence cortisol levels and also fat deposition and low muscle content in a

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[1] Nonstandard abbreviations: HPA, hypothalamo-pituitary adrenal; CBG, corticosteroid-binding globulin; GR, glucocorticoid receptor; WHR, waist-to-hip ratio; CV, coefficients of variation; PCR, polymerase chain reaction.

Table 1. Main characteristics of the 44 obese pre-menopausal women

Age (yr)	33.0 ± 1.3 [18.0–52.0]
Obesity parameters	
Body weight (kg)	107.3 ± 0.42 [72.8–141.0]
BMI (kg/m^2)	40.3 ± 0.8 [30.2–51.8]
TBFM (%)	54.9 ± 0.9 [42.3–66.6]
Visceral fat mass parameters	
WC (cm)	110.9 ± 2.0 [77.0–138.0]
WHR	0.87 ± 0.01 [0.66–1.00]
Metabolic parameters	
SBP (mm Hg)	123.0 ± 1.9
DBP (mm Hg)	76.0 ± 1.6
Plasma glucose (mM) [normal: 4.0–5.5 mM]	5.0 ± 0.08
LDL cholesterol (mM)	3.0 ± 0.1
Triglycerides (mM) [normal: 0.35–1.55 mM]	1.4 ± 0.1
HOMA	5.2 ± 0.5
HPA axis activity parameters	
Awakening salivary cortisol (nM)	
T0 (6:30 AM)	15.8 ± 1.2
T30	23.5 ± 0.28
T60	19.52 ± 0.42
AUC (10^2/nM/min)	1244 ± 86
Plasma cortisol at 8.00 AM (nM)	483.1 ± 21.3
ACTH at 8:00 AM (pg/mL)	10.4 ± 0.8
Post-DST (0.25 mg)	
Salivary cortisol (nmol/L)	10.1 ± 1.0
Plasma cortisol (nmol/L)	279.0 ± 23.3
CBG K_D (nM)	1.18 ± 0.04
Bmax (nM)	268.2 ± 13.2

Values are means ± SEM [range]. ACTH, adrenocorticotropic hormone; AUC, area under the curve; Bmax, maximal binding capacity; DBP, diastolic blood pressure; DST, dexamethasone suppression test; HOMA, homeostatis model assessment; LDL, low density lipoprotein; SBP, systolic blood pressure; TBFM(%), DXA measurement of total body fat mass; WC, waist circumference; WHR, waist-to-hip ratio.

pig intercross (11). In humans, CBG has also been suggested to participate in the constitution of obesity: patients with null mutation in the CBG gene tend to be obese and have a more rapid proliferation and greater differentiation of their preadipocytes compared to controls (12,13). Furthermore, serum CBG has been shown to be negatively correlated with BMI, waist-to-hip ratio (WHR), and some markers of metabolic syndrome in a human healthy population (14) and with markers of the metabolic syndrome in a population of obese women (15). However, CBG genetic polymorphisms have never been studied in human obesity.

For these reasons, we studied the influence of a CBG polymorphism on fat mass distribution in an obese female population extensively phenotyped for its HPA axis activity and reactivity. To minimize the influence of factors that may interfere with HPA axis activity, we applied stringent exclusion criteria (abnormal fasting glycemia, hypertension, depression, and any medical treatment). Phenotypic characteristics (obesity and visceral fat mass parameters, metabolic parameters, HPA axis activity parameters) for the 44 obese pre-menopausal women are shown in Table 1.

Three genotypes for the CBG gene, named after their base-pair sizes, were found: 86/86 (n = 29), 86/90 (n = 14), and 90/90 (n = 1). We first compared patients with genotypes 86/86 and 86/90 for obesity, metabolic, and HPA axis activity parameters using Student's unpaired t test. No significant difference was found between these two genotypes. Nevertheless, a trend toward higher diastolic blood pressure (78.1 ± 1.9 vs. 71.6 ± 1.6 mm Hg, p = 0.08), higher triglycerides (1.58 ± 0.15 vs. 1.12 ± 0.14 mM, p = 0.06)

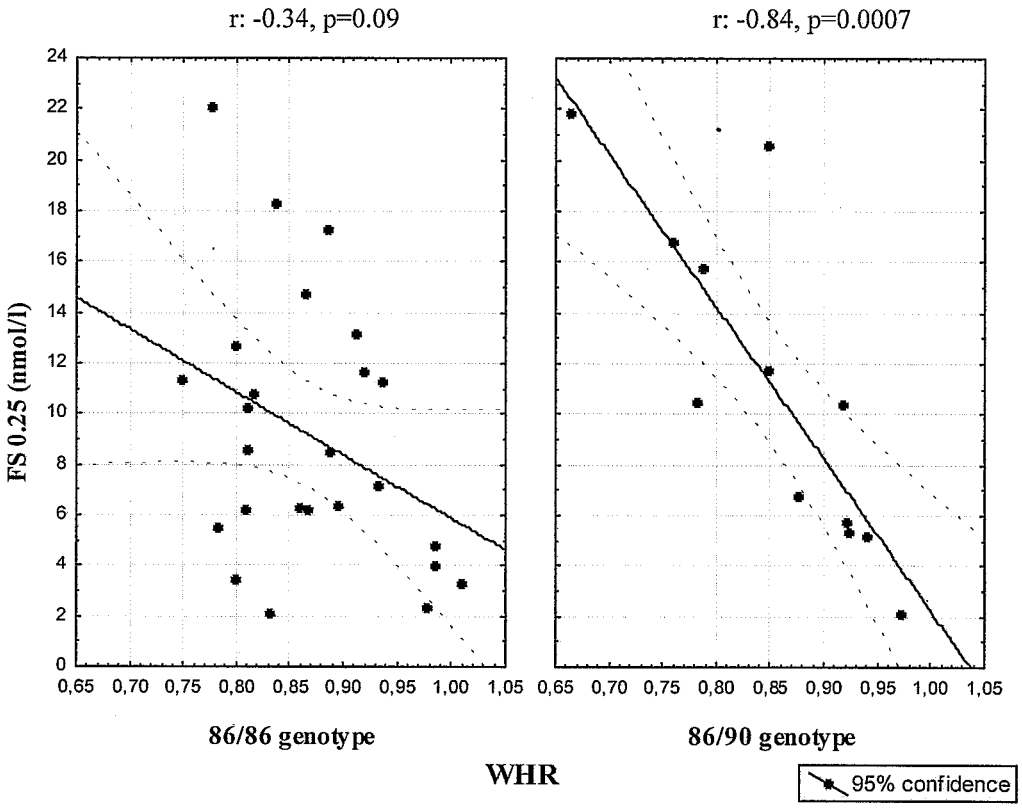

Figure 1: Correlations between salivary cortisol after dexamethasone (0.25 mg) suppressive test and WHR according to the CBG genotypes.

and higher cholesterol levels (5.3 ± 0.2 vs. 4.7 ± 0.2, $p = 0.06$) was found in patients 86/86 compared to patients 86/90. Because the population studied was small but stringently selected, we cannot exclude an important relationship between CBG polymorphism and the metabolic syndrome in a larger group of participants and a broader range of BMI including leaner as well as heavier subjects than those studied here. In a second step, we grouped the patients according to their genotype (86/86 or 86/90) and we searched for differences in correlations between HPA axis activity parameters and obesity or metabolic parameters. Because of the reduced number of women in each group, we looked for a difference between each group only through a stringent relationship ($p < 0.0001$) using Pearson's correlation coefficients. This situation was found only for correlation between salivary cortisol after dexamethasone suppression test (FS0.25) and WHR, in which patients with genotype 86/90 showed a greater correlation ($r = -0.84$, $p = 0.0007$) than patients with genotype 86/86 ($r = -0.34$, $p = 0.09$; difference between two correlation coefficients, $p = 0.02$) (Fig 1). In the genotype 86/90, the more the glucocorticoid sensitivity, the more the abdominal fat distribution. This relation was weaker in the genotype 86/86. Thus, CBG polymorphism could allow distinguishing two different populations, with one in which glucocorticoid sensitivity on Type II receptors, as measured by the dexamethasone suppression test, participates narrowly in fat mass distribution. The absence of difference in the binding capacity of CBG for cortisol between the CBG genotypes could be explained by the size of the population studied or because CBG expression could be modified specifically in adipose tissue and pituitary in the 86/90 genotype, leading to different relationships between glucocorticoid sensitivity and fat deposition. Nevertheless, for evident ethical reasons, we have no data for this last hypothesis regarding CBG mRNA expression levels in peripheral tissues.

To gain more insight into the involvement of the HPA axis in fat deposit, we also studied the glucocorticoid receptor gene polymorphism. Significant results are summarized in Table 2. There was an association between the BclI polymorphism and both awakening salivary cortisol and postdexamethasone suppressive test salivary and plasma cortisol. Patients with the BclI +/+ variant presented lower salivary cortisol level than the two other genotypes at awakening (BclI +/−) or 1 hour after awakening (BclI −/−). Patients with the BclI −/− variant showed less negative feedback than the two other variants, with higher postdexamethasone salivary cortisol concentration. The change we found for salivary cortisol is in accordance with previous studies on the BclI polymorphism of the glucocorticoid

Table 2. Significant associations between HPA axis activity and BclI polymorphisms

	BclI −/− (n = 6)	BclI +/− (n = 21)	BclI +/+ (n = 15)	p	p*
Awakening salivary cortisol (nML)					
T0 (6:30 AM)	15.9 ± 2.4	18.5 ± 2.1	12.2 ± 1.2†	NS	0.033
T30	28.4 ± 3.5	26.2 ± 3.3	17.8 ± 1.6	NS	0.050
T60	27.9 ± 4.4	19.9 ± 1.6	15.5 ± 2.3‡	0.013	0.012
Post awakening salivary cortisol (AUC)	1509 ± 185	1366 ± 137	956 ± 94	0.039	0.024
Post-dexamethasone suppression test (0.25 mg)					
Salivary cortisol (nM) 8 AM	17.9 ± 1.8	8.8 ± 1.5‡	8.7 ± 1.4‡	0.005	0.008
Plasma cortisol (nM) 8 AM	434.5 ± 76.3	235.7 ± 22.4	282.0 ± 41.2	0.020	0.046

Values are means ± SEM. $p^* = p$ after adjustment for BMI and DXA measurement of total body fat mass. NS, non-significant.
† Significant difference with BclI +/−.
‡ Significant difference with BclI −/−.

receptor (16). However, we failed to find any difference between BclI +/− and BclI +/+ genotypes for obesity parameters or parameters of the metabolic syndrome (null hypothesis not rejected with a p value >0.1 for all comparisons with Student unpaired t test). Furthermore, no difference in correlation was found between HPA axis activity parameters and obesity or metabolic parameters for the two most frequent genotypes (BclI +/+ and +/−). Finally, we examined the various CBG and GR genotype combinations for association. We did not detect any new association between these six genotypes and obesity, metabolic, or HPA axis activity parameters.

The strength of the current study is the extensive phenotyping of HPA axis activity and reactivity and the focus on pre-menopausal obese women with exclusion of patients presenting abnormal fasting blood glucose. However, these stringent inclusion parameters resulted in a population of small size that limits the interpretation of negative results but allows distinguishing subtle differences between genotypes. Overall, these data indicate that BclI polymorphism participates in the variability of HPA axis activity, but we found no association of GR gene polymorphism with obesity and metabolic parameters in our population. In contrast, we raise, for the first time, to our knowledge, the possibility that CBG gene polymorphisms may modulate the influence of the HPA axis on the fat mass distribution in obese pre-menopausal women.

Research Methods and Procedures

Subjects

Forty-four pre-menopausal non-smoking obese women (BMI >27 kg/m^2) of white origin referred for their obesity, gave written informed consent to participate in the study. The study had the full approval of the hospital ethics committee. Exclusion criteria were: clinical history of diabetes mellitus, alcohol addiction, hypertension, and therapy for any medical condition, including oral contraceptives (for a minimum of 3 months) and oral or inhaled steroids in the last 2 years and abnormal menstrual cycles. All had normal physical examinations apart from obesity, and normal hepatic, renal, and thyroid functions (normal basal thyroid-stimulating hormone value) as well as fasting glycemia <7 mM. According to the medical history, all had a stable weight (<3% variation over the last 3 months), and none had been dieting for at least 3 months before the study (<10% variation of caloric intake). Measurements were performed during the early follicular phase.

Study Design

As this study is a part of a more extended study aiming to characterize extensively hormonal and metabolic features associated with obesity in pre-menopausal women, we selected only relevant parameters of 1) obesity [weight, BMI, DXA (Lunar DPX-L, Lunar Radiation Corp., Madison, WI) measurement of total body fat mass (TBFM,%)]; 2) visceral fat mass (waist circumference, WHR); and 3) metabolic syndrome (systolic blood pressure, diastolic blood pressure, plasma glucose, low-density lipoprotein cholesterol, triglycerides, insulin sensitivity through homeostasis model assessment). Non-stimulated HPA axis activity was assessed by salivary cortisol at awakening (6:30 AM). Salivary cortisol response to awakening (area under the curve) was used to assess HPA axis reactivity. Pituitary sensitivity to glucocorticoids was explored using low-dose dexamethasone

suppression test (0.25 mg). Lastly, corticosteroid-binding globulin binding capacity was measured.

All of these explorations were conducted in the following order. The women were admitted to the department in the evening. The following day (Day 1), at 7:30 AM after an overnight fast, body weight and height, and waist and hip circumferences were recorded to the nearest 0.1 kg or cm. At 8:00 AM, blood was drawn to determine plasma biological tests. After this, body composition (DXA) was assessed. The following day (Day 2), a saliva sample (2 mL) was collected for determination of non-stimulated saliva cortisol at the time of awakening (6:30 AM) and every 30 minutes for 1 hour while subjects were asked to remain in a supine position, followed by standing up, and breakfast. On the late evening of Day 3, an overnight dexamethasone suppression test was performed at 11:00 PM, and all subjects were administered 0.25-mg dexamethasone orally. Blood and salivary samples were collected on the following morning (Day 4) at 8:00 AM for determination of serum cortisol and dexamethasone concentrations. The diet (beginning on Day 1 at lunch) consisted of 1500 kcal per day (55% carbohydrate, 30% fat, 15% protein), distributed in three meals (dinner between 6:00 and 6:30 PM).

Assays

Plasma cortisol concentrations were determined by a solid phase radioimmunoassays (Coat-a-count, Diagnostics Products corporation, Los Angeles, CA). The coefficients of variation (CVs) were: intra-assay 3–5% and inter-assay 6% to 8% for plasma cortisol concentrations between 130 and 650 nM. Saliva samples were diluted 10-fold with human desteroidated serum. Saliva cortisol was then extracted with dichloromethane (cortisol recovery >95%) and assayed after evaporation and resuspension of the dried extract in human desteroidated serum. The CVs were similar to the plasma assay CVs. All samples for cortisol determination were run in duplicates. The limits of detection of the assay were 29 nM and 1.9 nM for plasma and saliva, respectively. The binding capacity of CBG and its affinity for cortisol were measured at 4 °C by a solid phase assay using Concavalin A-Sepharose (Pharmacia Biotech, Buckinghamshire, UK).

Genetic Analysis

Leukocyte DNA was extracted from each subject using Nucleon BACC 2 kit (Amersham, Little Chalfont, UK). Genomic DNA (50 ng) was amplified by polymerase chain reaction (PCR) in a total reaction volume of 20 μL containing 0.5 μM of both the forward and the reverse primers, 2 nM of each of the four dNTPs, 1.5 $MgCl_2$, PCR buffer and 0.4U *Taq* polymerase, (Promega, Madison, WI). The PCRs were performed in 96-well microtitre plates. The PCR program was 1) 1 cycle 96 °C for 5 minutes; 2) 29 to 36 cycles 92 °C for 40 seconds, 55 to 60 °C for 1 minute, 72 °C for 30 seconds; 3) 1 cycle 72 °C for 2 minutes.

CBG Gene Analysis. The CBG polymorphism analyzed was a $(GTTT)_n$ repeat, located within intron 1 of the CBG gene and amplified with the following primers (forward, 5′-ACTCCAGCTTGGGCAACAACA-3′; reverse, 5′-CTGTGCATTTTTATATGGCTGGG-3′) (17). Alleles were visualized with ethidium bromide staining on a 3% agarose gel (Kalys, Roubaix, France).

Glucocorticoid Receptor Gene Analysis. Restriction fragment length polymorphism analysis was carried out to determine GR genotypes. Primer sequences surrounding the known BclI restriction site in the intron 2 (forward, 5′-GCAGTGAACAGTGTACCAG-3′; reverse 5′-TTTATCTGAATTGGGGATG-3′) were derived from the nucleotide sequence available in GenBank (accession # NT_ 006,489) for PCR amplification. The PCR products were digested with 1 unit of BclI enzyme (Promega) at 50 °C for 2 hours. BclI cleaves the 385 bp PCR product into two fragments (85 bp and 299 bp) that were visualized with ethidium bromide staining on a 3% agarose gel (Kalys, Roubaix, France).

Statistical Analysis

Data are presented as mean ± SEM. All phenotypic data listed in Table 1 were tested for association with each genotype. Student's unpaired *t* test was used to compare results between two genotypes. When more than two genotypes had to be compared, analyses were carried out with one-way ANOVA followed by least significant difference post hoc tests, adjusted for BMI and total body fat mass. The p value <0.05 was considered to be significant. We compared the relationships of HPA axis activity parameters and obesity or metabolic parameters for each genotype using Pearson's correlation coefficients. Because of multiple comparisons, we looked only for stringent correlation and $p < 0.001$. Statistical significance of differences between two correlation coefficients was calculated using "Difference between two correlation coefficients" tool provided in Statistica 6. The p value <0.05 was considered to be significant.

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