A Polymorphism in the Regulatory Region of the Corticotropin-Releasing Hormone Gene in Relation to Cortisol Secretion, Obesity, and Gene–Gene Interaction

Roland Rosmond, Monique Chagnon, Claude Bouchard, and Per Björntorp

In recent years, a considerable body of evidence has emerged regarding the pathogenic role of cortisol in abdominal obesity. The regulation of the corticotropin-releasing hormone (CRH) gene might play an essential role because it is the primary hypothalamic neuropeptide involved in the control of adrenal secretion of cortisol. Therefore, we examined the hypothalamic-pituitary-adrenal function by repeated salivary samples for the assessment of cortisol as well as other endocrine, anthropometric, metabolic, and circulatory variables in middle-aged Swedish men (n = 284). With the restriction enzyme *Xmn*I, a variant in the 5′-flanking region of the CRH gene was identified (T255G). The observed genotype frequencies were 89.9% and 9.7% for T/T and T/G, respectively. Only 1 subject was homozygous for the rare allele (0.4%; G/G). The results showed that the *Xmn*I polymorphism of the CRH gene is not associated with an altered cortisol-secretory pattern or sensitivity to glucocorticoids or with obesity and its related metabolic and circulatory perturbations. However, when the interaction effect between a previously described *TthIII*I glucocorticoid-receptor gene polymorphism and the present *Xmn*I CRH polymorphism was investigated, the cortisol levels before and during physiologic stress and the total diurnal cortisol secretion were significantly increased among subjects who were carriers for both variants. From these results, we conclude that an abnormal production rate of the CRH gene product in the presence of an inadequate glucocorticoid receptor density might lead to elevated cortisol levels.

CORTICOTROPIN-RELEASING hormone (CRH) is a 41–aminoacid peptide.[1] It is the primary hypothalamic neurohormone involved in the control of pituitary secretion of adrenocorticotropic hormone (ACTH), which stimulates cortisol secretion from the adrenal glands.[1] CRH is synthesized by neurons of the hypothalamic paraventricular nucleus.[2] CRH is also widely distributed throughout the central nervous system and in peripheral tissue such as the adrenal medulla,[3] testis,[4] gastrointestinal tract,[5] and placenta.[6,7]

Over the last decade, the catalog of disorders in which cortisol is believed to play a pathogenic role has grown exponentially.[8-11] Recent advances in this field have provided new insights into the pathogenesis of abdominal obesity, and a number of studies indicate that cortisol is associated with this condition.[12-21]

The presence of a continuously changing and sometimes threatening external environment sensitively activates the hypothalamic-pituitary-adrenal (HPA) axis to release cortisol.[22] Several factors that elicit such stress reactions have been described consistently in both men and women with abdominal obesity.[23-26] The level of stress response of the HPA axis is determined by hypothalamic regulators, including CRH, and the sensitivity of the negative glucocorticoid feedback.[1,22,27]

Recently, a restriction-length polymorphism in the 5′-flanking region of the CRH gene has been described.[28] DNA sequencing analysis has made it possible to identify a T→G base substitution that leads to the loss of an *Xmn*I site at position 255 of the Genbank entry X67661.[28] The human CRH gene has been localized to chromosome 8q13.[29] A mutation in regulatory sequences may result in an abnormal production rate of an otherwise normal CRH gene product. Because subjects with abdominal obesity often have an increased pituitary response to CRH,[12,19] this polymorphism of the CRH gene could be associated with this altered pituitary responsiveness. Interest in this hypothesis prompted the present study in a cohort of Swedish men. We investigated whether this *Xmn*I polymorphism of the CRH gene is associated with an altered cortisol-secretory pattern as well as obesity and its related metabolic and circulatory abnormalities. Furthermore, we examined the potential interactions between the *Xmn*I polymorphism of the CRH gene and 2 previously described variants of the glucocorticoid receptor gene (GRL) on chromosome 5.[30,31]

SUBJECTS AND METHODS

For the present study, we recruited subjects from an ongoing cohort study of men born in 1944.[14,15] The study was initiated in 1992. Based on self-measured waist-to-hip ratio (WHR), the following 3 subgroups, each of 150 men, were selected for further studies: those with the lowest ($\leq$0.885) and the highest ($\geq$1.01) values and those with values around the arithmetic mean (0.94 to 0.96). We examined these men in 1995 at age of 51 years, and 284 (63%) volunteered to participate in this second phase. All men gave written informed consent before participating in the study, which was approved by the Göteborg University ethics committee.

Salivary cortisol was measured repeatedly over a random working day, including morning and evening cortisol measurements as well as measurements of levels before, during, and after a standardized lunch.

From the Department of Heart and Lung Diseases, Göteborg University, Göteborg, Sweden, and the Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, LA.

Submitted October 19, 2000; accepted February 5, 2001.

Supported by grants from the Swedish Medical Research Council (K97-19X-00251-35A) and the Pennington Biomedical Research Center. R. Rosmond is the recipient of a postdoctoral fellowship at the Pennington Biomedical Research Center from the Henning and Johan Throne-Holst Foundation.

Address reprint requests to Roland Rosmond, MD, PhD, Department of Heart and Lung Diseases, Sahlgrenska University Hospital. S-413 45 Göteborg, Sweden.

0026-0495/01/5009-0005$35.00/0

doi:10.1053/meta.2001.25598

The arithmetic mean of all the cortisol measurements was calculated, providing an estimate of the total diurnal cortisol secretion. In addition, an overnight low-dose (0.5 mg) dexamethasone suppression test was performed at home with cortisol assayed in saliva. The details of these procedures have been published previously.[15]

Anthropometric measurements included body mass index (kg/m^2), WHR, and abdominal sagittal diameter (cm).[15] Endocrine measurements besides cortisol included testosterone, insulin-like growth factor I, and leptin as previously described in detail.[14,15]

Insulin, glucose, triglyceride, and total, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol levels; systolic and diastolic blood pressures; and heart rate were measured in the overnight fasting state as detailed previously.[14,15]

Genotyping was performed on leukocyte DNA. Polymerase chain reaction (PCR) amplification of the 5′-flanking region of the CRH gene was carried using primers described previously.[28] The 818-bp PCR products were digested with *Xmn*I. The frequent allele (T) contains the intact *Xmn*I recognition site and when digested produces 2 fragments at 245 bp and 573 bp (G).

All statistical analyses were performed using the SAS System for Windows, release 6.12 (SAS Institute Inc, Cary, NC). Differences between genotypes were assessed by use of the Mann-Whitney *U* test. Analyses of potential interactions between gene markers were performed within the framework of general linear models (PROC GLM). All *P* values were adjusted for multiple (simultaneous) tests using Hommel's modifications of the Bonferroni procedure.[32]

RESULTS

The frequency of allele T was 0.95 and 0.05 for allele G. The observed genotype frequencies were 89.9% and 9.7% for T/T and T/G, respectively. Only 1 subject was homozygous for the rare allele (0.4%; G/G). Genotype frequencies were in a Hardy-Weinberg equilibrium.

Table 1 shows the results for the differences in salivary cortisol measurements by genotype of the *Xmn*I polymorphism of the CRH gene. Differences in salivary cortisol variables between the 2 genotype groups were not significant. Furthermore, there were no statistically significant differences in anthropometric, endocrine, metabolic, and circulatory measurements between the 2 genotypes (Table 2).

Next, we investigated the potential interaction effects with previously described *Bcl*I and *TthIII*I GRL variants.[30,31] Carriers for both the *Bcl*I variant of the *GR*L (4.5-kb) and the *Xmn*I rare allele (G) of the CRH gene had significantly lower testosterone ($P = .020$) than noncarriers and carriers of only 1 of the variants. No other significant interaction effects emerged.

Table 3 shows the results of the interaction effects between the *Tthlll*I *GRL* marker and the *Xmn*I CRH marker. The *Tthlll*I allelic frequency was 0.30 and 0.70 for the 3.8- and 3.4-kb allele, respectively.[31] Carriers of both the *Tthlll*I variant of the *GRL* (3.8-kb) and the *Xmn*I rare allele of the CRH gene had significantly higher cortisol levels at 11:45 AM and 30, 45, and 60 minutes after a standardized lunch. These subjects also had significantly higher total diurnal cortisol level.

Table 1. Differences in Salivary Cortisol Measurements by Genotype of the CRH Gene Polymorphism

	Genotype	
	T/T (n = 241)	T/G (n = 26)
Cortisol level (nmol/L) in the morning	14.8 (7.5)	14.7 (6.1)
Cortisol level (nmol/L) at 11:45 AM	7.0 (4.2)	9.4 (11.0)
Cortisol level (nmol/L) 30 min after lunch	8.1 (7.3)	10.0 (12.6)
Cortisol level (nmol/L) 45 min after lunch	7.4 (4.8)	8.6 (9.2)
Cortisol level (nmol/L) 60 min after lunch	6.7 (4.4)	8.5 (10.4)
Cortisol level (nmol/L) 5:00 PM	4.8 (2.4)	5.3 (2.9)
Cortisol level (nmol/L) before bedtime	3.3 (4.5)	3.5 (3.5)
Total diurnal cortisol level (nmol/L)	7.3 (3.2)	8.6 (6.3)
Dexamethasone suppression test (nmol/L)	12.1 (5.4)	11.8 (5.1)

NOTE. Values are given as mean (SD). All *P* values are >.20.

Table 2. Differences in Anthropometric, Endocrine, Metabolic, and Circulatory Measurements by Genotype of the CRH Gene Polymorphism

	Genotype	
	T/T (n = 241)	T/G (n = 26)
Body mass index (kg/m^2)	26.1 (3.9)	26.3 (3.9)
WHR	0.9 (0.1)	0.9 (0.1)
Abdominal sagittal diameter (cm)	22.6 (3.6)	23.1 (3.8)
Testosterone (nmol/L)	19.8 (5.5)	19.4 (5.1)
Insulin-like growth factor I (μg/L)	205.1 (65.4)	211.7 (62.9)
Leptin (μg/L)	6.2 (4.3)	5.7 (4.1)
Fasting insulin (mU/L)	12.9 (11.4)	10.3 (5.2)
Fasting glucose (mmol/L)	4.5 (1.0)	4.8 (1.0)
Triglycerides (mmol/L)	1.8 (1.1)	1.9 (1.0)
Total cholesterol (mmol/L)	6.2 (1.1)	6.2 (1.0)
HDL cholesterol (mmol/L)	1.3 (0.3)	1.3 (0.3)
LDL cholesterol (mmol/L)	4.1 (1.0)	4.2 (0.9)
Systolic blood pressure (mm Hg)	129.0 (17.1)	133.8 (20.2)
Diastolic blood pressure (mm Hg)	83.4 (10.5)	84.6 (10.8)
Heart rate (beats/min)	69.0 (10.6)	67.5 (10.3)

NOTE. Values are given as mean (SD). All *P* values are >.20.

DISCUSSION

Our study indicates that a T→G nucleotide substitution in the 5′-flanking region of the CRH gene, resulting in the loss of a *Xmn*I restriction site at position 255, by itself is not associated with an altered cortisol-secretory pattern or sensitivity to glucocorticoids or with obesity and its related metabolic and circulatory perturbations. In the cohort examined herein, the *Xmn*I allelic frequencies were 0.95 and 0.05 respectively, which are identical to a previous report in Caucasians.[28] However, as implied by a recent study, the allele frequency for this polymorphism in the CRH promoter region might be extremely divergent between populations.[33]

The men examined were selected from an ongoing cohort study, and 80% volunteered to participate in the first part of the study. The second part, which was laboratory-based, attracted fewer participants (63%), but there were no differences between respondents and nonrespondents in terms of prevalence of hypertension, diabetes mellitus, myocardial infarction, stroke, and angina pectoris, or in education level, housing conditions, and smoking and alcohol habits.[14,15] Therefore, we

Table 3. Differences in Salivary Cortisol Measurements Between Carriers for the *Xmn*I Rare Allele of the CRH gene (G) and the *TthIII* I variant of the *GRL* (3.8 kb)

	G Carriers and 3.8-kb Carriers (n = 12)	G Carriers and 3.4-kb Carriers (n = 14)	T Carriers and 3.8-kb Carriers (n = 128)	T Carriers and 3.4-kb Carriers (n = 113)	*P*
Cortisol level (nmol/L) at 11:45 AM	13.0 (15.8)	6.5 (2.6)	6.9 (3.0)	7.0 (5.4)	.006
Cortisol level (nmol/L) 30 min after lunch	14.2 (18.2)	6.5 (1.8)	8.0 (5.1)	8.3 (9.4)	.025
Cortisol level (nmol/L) 45 min after lunch	11.5 (13.3)	6.3 (2.1)	7.4 (5.1)	7.4 (4.5)	.034
Cortisol level (nmol/L) 60 min after lunch	11.7 (15.0)	5.7 (2.0)	6.8 (3.8)	6.5 (5.1)	.016
Total diurnal cortisol level (nmol/L)	10.8 (8.9)	6.7 (2.1)	7.6 (3.9)	6.9 (2.1)	.045

NOTE. Values are given as mean (SD).

believe the participating men are representative of men at this age in the city of Göteborg, Sweden.

Assessment of the HPA axis function in the present study included measurements of salivary cortisol levels under basal conditions and during challenge by food intake or suppression by dexamethasone. The assessment of cortisol in saliva is specific for the detection of unbound, free cortisol, and the concentrations of cortisol in saliva is independent of the saliva flow.[34] The test is sensitive enough to measure cortisol levels in normal subjects and to distinguish normal secretory patterns from hypocortisolism and hypercortisolism.[35,36] A recent study has shown that an increase in salivary cortisol is reliably elicited by a standard protein-rich meal.[37] Moreover, cortisol in saliva accurately reflects the free fraction of cortisol in plasma,[34] which has also been confirmed in our laboratory ($r > .90$, unpublished). From a practical point of view, assessment of cortisol from saliva samples represents less of a burden for subjects. Such samples are also simple for the hospital staff to process and analyze.

We have recently shown that a *Bcl*I restriction fragment length polymorphism in intron 1 of the *GRL* is associated with decreased sensitivity to elevated postprandial cortisol secretion and with several cardiovascular risk factors, including abdominal obesity.[30] In addition, a variant in the 5′-flanking region of the *GRL*, identified with the restriction enzyme *TthIII*I, has been found to be associated with elevated basal cortisol secretion.[31] Consequently, we investigated the potential interaction effects between the *Xmn*I CRH gene polymorphism and the *Bcl*I as well as the *TthIII*I GRL polymorphism.

When the interaction effect between the *TthIII*I GRL polymorphism and the *Xmn*I CRH polymorphism was investigated, the cortisol levels before and during lunch as well as total diurnal cortisol secretion were significantly increased among subjects who were carriers for both variants (Table 3).

Besides the stimulatory effect exerted by CRH, the cortisol secretion is also regulated by the inhibitory feedback action of cortisol, which is mediated through specific glucocorticoid receptors (GR).[1,22,27] Based on the results of the present study, one could speculate that an abnormal production rate of the CRH gene product in the presence of inadequate GR density results in elevated cortisol levels. Taken together, we tentatively interpret the results as follows. DNA sequence variation in the 5′-flanking region of the CRH gene results in an abnormal production rate of an otherwise normal CRH gene product. The resulting increase in CRH concentrations affects the secretory end product of the HPA axis, cortisol, which is kept above optimal range. Because the *TthIII*I polymorphism is localized in the promoter region of the *GRL*,[38] it is more likely to be involved in the regulation of GR density than the production of a GR that has low affinity for glucocorticoids and/or is unstable during thermal activation.

A shortcoming of the present study is the absence of CRH measurements. Peripheral plasma concentrations of hypothalamic peptides are lower than those in the brain, and many investigators have found that peripheral plasma CRH does not correlate with plasma ACTH or cortisol levels. However, the elevated total diurnal cortisol level (Table 3) indicates that CRH concentrations are most likely increased. Only future work will show whether the interaction effects described herein reflect a physiologic role of the *Xmn*I CRH gene polymorphism in the regulation of cortisol secretion.

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