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Gender difference in adrenal sensitivity to ACTH is abolished in type 2 diabetes

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Abstract

Objective: Dysfunction of the hypothalamus–pituitary–adrenal (HPA) axis has been implicated in type 2 diabetes (T2D). The aim of this study was to investigate the impact of T2D and gender on the HPA axis.

Methods: Synthetic ACTH (1 μg) was administered to 21 subjects with T2D (age 62 (54–70) years, 11 men/ten women, HbA1c 49±2 mmol/mol, treated with diet or oral antidiabetic drugs) and 38 controls (age 58 (41–67) years, 20 men/18 women). Fasting basal B-glucose, serum cortisol, insulin, IGF1 and IGFBP1 concentrations were measured, and sampling for all but IGF1 was repeated 30, 60, and 90 min after ACTH injection. Patients took 0.25 mg dexamethasone at 2200–2300 h and returned the next morning for the measurement of serum cortisol concentration.

Design: Cross-sectional study.

Results: Patients with T2D had similar fasting serum cortisol, IGF1 and IGFBP1 concentrations; however, serum cortisol concentration after administration of dexamethasone did not differ between the groups. Healthy women exhibited higher peak cortisol levels compared with healthy men (675±26 vs 582±21 nmol/l, $P=0.014$), while the peak levels were equally high in men and women with T2D, resulting in a higher peak level in men with T2D compared with healthy men (691±42 vs 582±21 nmol/l, $P=0.024$). Serum cortisol concentration after administration of dexamethasone did not differ between the groups, nor did IGF1 and IGFBP1.

Novelty of the findings: Some studies have previously indicated disturbed regulation of the hypothalamus–pituitary–adrenal (HPA) axis in subjects with type 2 diabetes (T2D); however, much remains unknown in this area. To the best of our knowledge, this is the first study to show that the gender difference in the adrenal response to ACTH (with greater reactivity in women) is abolished in T2D. While the clinical implications cannot be determined by this paper, it is known that gender differences exist in the pathogenesis and complications of T2D. Thus, our findings suggest that further research into gender differences in the HPA axis is warranted.

Conclusions: Gender differences in adrenal response to ACTH were abolished in T2D. Men with T2D had a higher peak cortisol compared with controls. Further studies are needed to elucidate the clinical implications.

Key Words
- diabetes
- cortisol
- HPA axis
- IGF1
- IGFBP1
- gender

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Introduction

Along with progressive β-cell failure and insulin resistance, patients with type 2 diabetes (T2D) display disturbed regulation of cortisol and insulin-like growth factor 1 (IGF1) secretion (1). Especially regarding the hypothalamus–pituitary–adrenal (HPA) axis, studies have shown discrepant results, reporting both increased and decreased activation (2).

Cortisol has effects opposite to those of insulin on glucose metabolism, decreasing glucose uptake and increasing gluconeogenesis (1, 2, 3). However, elevated levels of both cortisol and insulin stimulate accumulation of visceral adipose tissue (4). The phenotypes of hypercortisolism and T2D are similar, with insulin resistance, visceral obesity, hypertension, and dyslipidemia (2). Some (5), but not all (6), studies have indeed shown increased production of cortisol, as well as blunted sensitivity to feedback inhibition. In obese non-diabetic subjects, basal and stimulated cortisol levels and sensitivity to feedback inhibition have been found to be normal (7, 8) or decreased (9).

These inconsistent results may be explained by gender differences in HPA axis regulation. Many previous studies have only included patients of one gender, or have not factored in gender in the analyses (7, 9). Younger, healthy women have increased adrenal response to physiological stress compared with men due to a stimulatory effect of estrogen on the HPA axis (10).

Increased glucocorticoid levels or HPA axis activity may furthermore suppress growth hormone (GH) secretion and the action of IGF1 in target tissues (11, 12). IGF1 is produced in the liver, under regulation by GH, insulin and nutritional status (13). It has effects similar to those of insulin and improves insulin sensitivity (13). IGF1 levels may be low in subjects with T2D (14, 15). IGF binding protein 1 (IGFBP1) binds IGF1 and functions as a transport protein, as well as regulating IGF1 bioavailability (16). Insulin inhibits IGFBP1 production via reduced gene transcription (17). Because of its acute regulation by insulin, IGFBP1 is a marker of hepatic insulin sensitivity (18). Cortisol increases *IGFBP1* gene transcription; however, this effect is less potent than the inhibiting effect of insulin (19).

Most studies have used the standard 250 μg adrenocorticotropic hormone (ACTH) stimulation test and the 1 mg dexamethasone test of feedback inhibition to evaluate the HPA axis. However, the 250 μg test induces supraphysiological ACTH levels, and may therefore not be sensitive enough to reveal more discrete disturbances in the HPA axis (20). The low-dose, 1 μg test provides a more 'physiological' stimulation of the adrenal cortex compared with the standard dose and correlates better with the 'golden standard' insulin tolerance test (20). In most subjects, serum cortisol is completely suppressed after administration of 1 mg dexamethasone, while 0.25 mg gives a smaller although significant reduction (21). This implies that the low-dose test may be suited for detecting discrete disturbances in the sensitivity of the HPA axis to feedback inhibition (22).

In summary, while many studies have reported disturbed regulation of the HPA axis and IGF1 in metabolic diseases, much remains unknown, especially regarding T2D. Standard methods were used to examine the lack of sensitivity of the HPA axis, and many studies have not accounted for possible gender differences.

Aims

The purpose of this study was to test the hypothesis that patients with T2D have increased serum cortisol levels after administration of low-dose ACTH or low-dose dexamethasone, as well as lower serum IGF1 levels compared with healthy controls. We furthermore aimed to investigate whether these parameters are affected by gender in healthy subjects and in subjects with T2D.

Materials and methods

A total of 59 subjects were enrolled in the study: 21 with T2D (11 men and ten women) and 38 healthy controls (20 men and 18 women). Participants were recruited primarily from a database of subjects previously enrolled in or screened for studies at the Department of Endocrinology, Diabetes and Metabolism at the Karolinska University Hospital, as well as by advertisement. The study was approved by the local ethics committee. Informed consent was obtained from all patients before inclusion in the study. Patients with T2D were allowed to take oral antidiabetic drugs (OADs). Exclusion criteria were insulin or glucocorticoid therapy. One male patient with T2D was excluded from the analyses before and after the dexamethasone test (see below), as he had been started on basal insulin between the time of the ACTH and dexamethasone tests. Control subjects were considered healthy based on patient history and fasting plasma glucose levels.

For all visits, subjects were instructed to fast after 2200 h the previous evening and refrain from using tobacco in the morning of the test day. Each subject was interviewed regarding medical history. Weight, height,

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and waist and hip circumference were recorded, and BMI was calculated as weight (kg)/height (m^2).

On the first visit, a low-dose ACTH test was performed. Subjects rested throughout the test. A cannula was inserted into an antecubital vein and blood was drawn for the analysis of blood glucose and serum cortisol, insulin, IGF1 and IGFBP1. The low-dose ACTH solution was prepared by removing 1 ml from a 50 ml bottle of NaCl 9 g/l, and then adding 1 ml of 0.25 g/l solution synthetic ACTH (Synacthen; Novartis) to the 50 ml bottle, resulting in a concentration of 250 μg/50 ml = 5 mg/l. A 1 μg injection was prepared by drawing up 0.2 ml of the 5 mg/l solution, and then 0.8 ml of pure NaCl solution. The injection was administered at 0800 h. Blood was drawn 30, 60, and 90 min after the injection, for repeated analyses of glucose, insulin and IGFBP1. The cannula was flushed with physiological NaCl solution after each sampling.

Of the total study population, 32 subjects (eight with T2D and 24 healthy controls) made an additional visit for a placebo test with NaCl; the test was performed in a random order. Serum cortisol level is normally highest early in the morning, and then decreases gradually during the daylight hours. We expected to provoke a temporary increase in serum cortisol level with Synacthen but not with placebo. The purpose of this test was to exclude confounding activation of the HPA axis due to the stress. For the placebo tests, the same test protocol was followed as for the ACTH test, but 10 ml physiological NaCl solution was injected instead of Synacthen. The procedure was blinded to the patient.

Only the first 32 subjects were tested with ACTH and NaCl tests. They were called back for a low-dose dexamethasone test and new basal cortisol was drawn, whereas in patients who were included later the ACTH test provided basal cortisol. After basal sampling, all subjects were given a capsule of 0.25 mg dexamethasone, which they were instructed to take between 2200 and 2300 h in the evening before their second visit. On that occasion, they returned to the testing facility at 0800 h, and blood was drawn for measurement of serum cortisol.

Blood glucose was analyzed from whole blood within 30 min from sampling using YSI 2300 Stat Plus apparatus (YSI Life Sciences, Yellow Springs, OH, USA).

HbA1c was measured from capillary whole blood using a spectrophotometric technique (DCA Vantage; Siemens, Munich, Germany). Results are dually reported as NGSP (%) and IFCC (mmol/mol) (NGSP 2010, http://www.ngsp.org/convert1.asp).

Serum cortisol was analyzed using Roche Modular apparatus (Roche Diagnostics Scandinavia). The total coefficient of variation (CV) was 2.5% at 544 nmol/l and 2.1% at 855 nmol/l.

The remaining tests for insulin, IGF1 and IGFBP1 were centrifuged for 15 min at 1700 ***g***, 15 °C, and the supernatant stored at −80 °C pending analysis in the same run.

Serum insulin was measured by RIA (Pharmacia insulin RIA 100, Pharmacia Diagnostics). The interassay CV was <5.8% and the intra-assay CV <5.4%.

Serum IGF1 was determined by an in-house RIA after separation of IGFs from IGFBPs (23). Cross-reactivity with IGFBP2 and IGFBP3 was <0.5 and <0.05% respectively. To minimize the interference of the remaining IGFBPs, des(1–3) IGF1 was used as radioligand. Serum levels of IGF1 decrease with age and are thus expressed as SDS = ((10logIGF1-observed + 0.00693 × age) − 2.581)/0.120 (24). The intra- and interassay CV were 4 and 11% respectively.

Serum IGFBP1 was also analyzed with an in-house RIA (25). The sensitivity of the RIA was 3 μg/l, and the intra- and interassay CV were 3 and 10% respectively.

Quantification of insulin resistance ▸ The homeostatic model of insulin resistance (HOMA-IR) was calculated as (fasting serum insulin × fasting blood glucose)/22.5 (26).

Statistical analyses

Statistical analyses were carried out using STATISTICA Software, version 10 (StatSoft, Tulsa, OH, USA). *P* values <0.05 were considered statistically significant. Variables are presented as means ± S.E.M. unless otherwise stated. Changes in variables after a test, compared with those before test, are designated as Δ. Normality of variables was tested using the Kolmogorov–Smirnov and Lilliefors tests. Differences between groups in variables that were normally distributed were analyzed using paired and unpaired *t*-tests, whereas variables that were not normally distributed were analyzed using the Wilcoxon and Mann–Whitney *U* tests. Repeated measurements were studied using repeated measures ANOVA.

Body composition has a well-established impact on the regulation of the HPA axis (1). Therefore, correlation analyses were performed between basal cortisol and BMI and waist circumference respectively, using Pearson's correlation coefficient. Multiple linear regressions were performed with selected sets of continuous and categorical variables, as described in the Results section. Variables were included in the multiple regression models if $P<0.10$.

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Table 1 Subject characteristics – subjects with type 2 diabetes (T2D) vs healthy subjects. Mean ± S.E.M. except for data measured in years, presented as median (range).

	T2D ($n=21$)	Healthy ($n=39$)	*P*
Age (years)	62 (54–70)	58 (41–67)	0.010
BMI (kg/m^2)	26.6 ± 0.7	26.7 ± 0.7	NS
Waist (cm)	99 ± 2	94 ± 3	0.022
B-glucose, basal (mmol/l)	6.5 ± 0.3	4.9 ± 0.1	<0.001
S-insulin, basal (mU/l)	22.4 ± 2.6	16.6 ± 1.2	0.012
HOMA-IR (mmol × mU)	6.6 ± 0.9	3.6 ± 0.3	<0.001
HbA1c (% (mmol/mol))	6.6 ± 0.18 (49 ± 2)		
T2D duration (years)	9 (1–19)		

HOMA-IR, homeostatic model assessment of insulin resistance; repa, repaglinide; SU, sulfonylurea.

Results

Subject characteristics

The T2D group had good metabolic control with a mean HbA1c of 6.6% DCCT (49 ± 2 mmol/mol). All 16 T2D patients on OADs had received metformin. In addition, eight also had received sulfonylurea or repaglinide (SU/repa) and/or other drugs as specified in Table 1. No patients had received SU/repa as monotherapy; five subjects were on diet alone. T2D patients had higher basal blood glucose ($P<0.001$; Table 2), serum insulin ($P=0.012$) and HOMA-IR ($P<0.001$) levels compared with controls. There were no gender differences in mean HbA1c or duration of T2D.

All pati ents were middle aged, although men with T2D were slightly older than male controls ($P=0.009$; Table 2). All groups were matched for BMI; however, the T2D group had higher waist circumference compared with the controls ($P=0.022$).

Among the healthy women, one still had regular menstruation, and one less regularly than previously. The remaining women were post-menopausal, based on patient history. Smoking status, menstruation, and treatment with hormone replacement therapy ($n=2$ healthy women for all) did not affect the outcome of the analyses.

Effect of ACTH stimulation compared with NaCl

After both ACTH and NaCl injection, glucose levels were unaffected, whereas serum insulin levels decreased (0 vs 90-min measurements; $P=0.033$ for ACTH, $P=0.044$ for NaCl in T2D patients, $P<0.001$ for ACTH, $P=0.012$ for NaCl in healthy subjects). Serum cortisol levels decreased after NaCl injection (for T2D patients from 409 ± 34 nmol/l (basal) to 260 ± 37 nmol/l after 90 min; $P=0.008$ for basal vs 90 min). For healthy subjects, cortisol decreased from 394 ± 23 nmol/l to 244 ± 15 nmol/l after 90 min; $P<0.001$), while it increased in all after ACTH.

Gender difference in adrenal response to ACTH in healthy subjects but not in T2D patients

After ACTH injection, healthy women had higher peak cortisol levels ($P=0.023$) compared with healthy men

Table 2 Subject characteristics – subjects with type 2 diabetes (T2D) vs healthy subjects, subdivided by gender. Mean ± S.E.M. except for data measured in years, presented as mean (range).

	T2D men ($n=11$)	T2D women ($n=10$)	*P* T2D men vs women	Healthy men ($n=20$)	Healthy women ($n=18$)	*P* healthy men vs women	*P* T2D vs healthy men	*P* T2D vs healthy women
Age (years)	63 (56–70)	60 (54–70)	NS	57 (41–67)	57 (41–64)	NS	0.009	NS
BMI (kg/m^2)	26.2 ± 0.8	27.0 ± 1.3	NS	26.5 ± 0.89	27.0 ± 1.1	NS	NS	NS
Waist (cm)	102 ± 3	95 ± 3	NS	100 ± 5	88 ± 4	0.006	NS	NS
B-glucose, basal (mmol/l)	6.7 ± 0.4	6.3 ± 0.4	NS	4.9 ± 0.1	4.8 ± 0.1	NS	<0.001	<0.001
S-insulin, basal (mU/l)	24.5 ± 4.2	20.0 ± 2.9	NS	15.9 ± 1.3	17.3 ± 2.0	NS	0.029	NS
HOMA-IR (mmol × mU)	7.5 ± 1.5	5.6 ± 0.7	NS	3.5 ± 0.3	3.9 ± 0.5	NS	0.002	0.023
T2D duration (years)	11 (2–19)	8 (1–11)	NS					
HbA1c (% (mmol/mol))	6.6 ± 0.18 (49 ± 2)	6.5 ± 0.18 (48 ± 2)	NS					
Metformin monotherapy	2	2						
Add-on SU/repa	6	2						
Add-on SU/repa + pioglitazone	1	0						
Add-on sitagliptin	2	0						
Add-on SU/repa + sitagliptin	1	0						
Add-on liraglutide	0	1						
Add-on acarbose	1	0						

Add-on, in addition to metformin therapy; HOMA-IR, homeostatic model assessment of insulin resistance.

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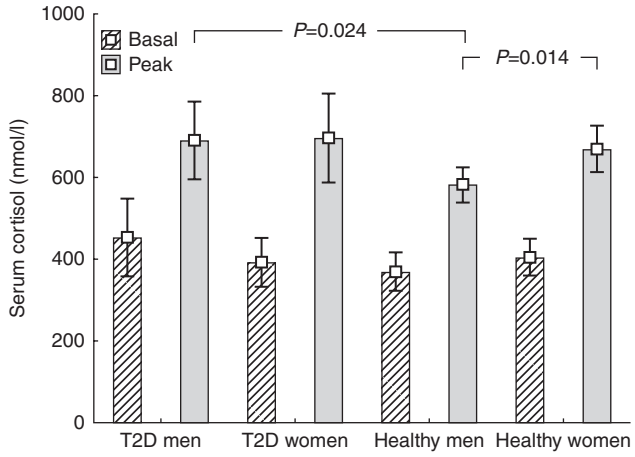

Figure 1
Serum cortisol before, and at peak after, 1 μg ACTH injection.

(Fig. 1 and Table 3). Despite similar basal cortisol levels, the T2D group had higher peak cortisol levels ($P=0.043$). This was due to higher peak cortisol levels in men with T2D compared with male controls ($P=0.024$), while peak levels did not differ between healthy and diabetic women. The incremental area under the curve for cortisol was also higher in healthy women than in men ($P=0.022$), while, as with peak cortisol levels, there was no gender difference in T2D patients. Both men and women with T2D had peak cortisol levels similar to those of healthy women. Basal but not peak cortisol levels correlated with BMI ($r=-0.461$, $P=0.004$) and waist circumference ($r=-0.467$, $P=0.003$) in healthy subjects but not in T2D patients.

Serum cortisol levels after administration of dexamethasone

The T2D and healthy groups did not differ in fasting cortisol levels after administration of dexamethasone (Table 4). Cortisol levels decreased in all four subgroups after administration of dexamethasone (Table 3). There were no differences in the magnitude of the decrease between the T2D and healthy groups, or by gender. However, those in the T2D group on metformin and SU/repa, compared with those without, had higher serum cortisol levels after administration of dexamethasone ($P=0.041$).

Factors affecting peak cortisol levels after ACTH injection

Multiple linear regressions were performed to assess the effects of gender, disease status (T2D or healthy), BMI, waist circumference and basal serum insulin on peak cortisol (Table 5; model 1). Waist circumference had the lowest impact, and also neared multicollinearity with BMI (correlation of regression coefficient 0.778). After its removal, only gender was significant ($P=0.045$; model 2). In a final model including basal insulin, gender and BMI (model 3), gender remained the only significant independent variable ($P=0.048$), although BMI showed a trend toward affecting peak cortisol levels negatively ($P=0.063$). However, none of these models had a high r^2, indicating that other factors not measured also affected peak cortisol levels.

IGF1 and IGFBP1

T2D patients and healthy subjects did not differ in fasting serum IGF1 or IGFBP1 levels, in spite of higher insulin levels in T2D patients.

Discussion

A novel finding in the present study was that the gender difference that exists in the adrenal response to ACTH in healthy subjects, with higher peak cortisol levels in

Table 3 Hormone levels, basal and after administration of 1 μg ACTH and 0.25 mg dexamethasone (dex) – healthy subjects vs T2D, subdivided by gender.

	T2D men ($n=11$)	T2D women ($n=10$)	*P* T2D men vs women	Healthy men ($n=20$)	Healthy women ($n=18$)	*P* healthy men vs women	*P* T2D vs healthy men	*P* T2D vs healthy women
S-IGF1, basal (S.D.)	0.4±0.5	0.2±0.3	NS	0.2±0.2	0.3±0.2	NS	NS	NS
S-IGFBP1, basal (μg/l)	37±4	34±5	NS	32±4	38±5	NS	NS	NS
S-cortisol, basal before ACTH (nmol/l)	451±43	391±26	NS	368±22	403±22	NS	NS	NS
S-cortisol, peak after ACTH (nmol/l)	691±42	696±47	NS	582±21	675±26	0.023	0.024	NS
Δ cortisol after ACTH (nmol/l)	240±31	305±42	NS	214±23	267±17	NS	NS	NS
S-cortisol, basal before dex (nmol/l)	473±40	423±43	NS	409±22	380±22	NS	NS	NS
S-cortisol, after dex (nmol/l)	321±42	244±24	NS	280±21	240±22	NS	NS	NS
Δ cortisol after dex (%)	−32.0±7.3	−40.4±5.8	NS	−32.2±2.9	−39.7±5.1	NS	NS	NS

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Table 4 Hormone levels, basal after administration of 1 μg ACTH and 0.25 mg dexamethasone (dex) – subjects with type 2 diabetes (T2D) vs healthy subjects. Mean ± S.E.M.

	T2D ($n=21$)	Healthy ($n=39$)	*P*
S-IGF1, basal (S.D.)	0.3 ± 0.3	0.3 ± 0.1	NS
S-IGFBP1, basal (μg/l)	36 ± 3	35 ± 3	NS
S-cortisol, basal before ACTH (mmol/l)	424 ± 27	385 ± 16	NS
S-cortisol, peak level after ACTH (nmol/l)	693 ± 31	624 ± 18	0.043
Δ cortisol from basal to peak level after ACTH (nmol/l)	269 ± 26	239 ± 15	NS
S-cortisol, basal before dexamethasone (nmol/l)	448 ± 29	404 ± 15	NS
S-cortisol, after dexamethasone (nmol/l)	283 ± 25	261 ± 15	NS
Δ cortisol from basal after dexamethasone (%)	−36.2 ± 4.6	−35.6 ± 2.9	NS

women compared with men, was abolished in patients with T2D. This was due to increased peak cortisol levels in men with T2D.

Greater reactivity of the HPA axis to physiological stress has previously been shown in healthy women compared with men, potentially due to estrogen effects (27). However, the healthy female subjects in this study retained a higher adrenal response to ACTH compared with healthy men despite being predominantly postmenopausal, suggesting additional explanations other than estrogen. The control test with NaCl showed that the increase in cortisol levels after ACTH injection was due to the ACTH *per se*, not due to stress during the procedure.

Potential factors explaining the difference between healthy and diabetic men in adrenal response to ACTH are age, body composition, medications, and insulin levels. The age difference is unlikely accountable for the higher cortisol response to ACTH in the T2D group, as both were middle aged and HPA axis reactivity is unaffected by ageing (28). Abdominal adiposity has been linked with increased cortisol response to physiological and psychological stressors (1, 29), such as was observed in the T2D patients in the present study. However, the two male groups were matched for waist circumference, eliminating this as a confounder explaining the increased HPA reactivity in men with T2D. It cannot be excluded that medications, which varied between the subjects, may have had effects on the activity of the HPA axis.

Supraphysiological insulin levels acutely increase ACTH and cortisol in healthy subjects (30). As the T2D patients in our study had higher fasting serum insulin levels and waist circumference compared with the healthy subjects, multiple regression models were designed as outlined above, using peak cortisol as the dependent variable and gender, disease status, BMI and basal insulin as independent variables. While these analyses confirmed an impact on gender, basal insulin levels were not related to peak cortisol levels in any model.

The degree of feedback inhibition of cortisol after administration of 0.25 mg dexamethasone was of comparable magnitude to that seen in other studies utilizing the same method (22). It was similar in both T2D and healthy subjects, and in men and women. The T2D patients in this study had good glycemic control and moderate insulin resistance, as suggested by low HbA1c, and moderately elevated insulin levels. The effects of OADs on cortisol responses to ACTH and dexamethasone may either be direct or reflect that patients requiring additional pharmacological therapy have more advanced disease, in turn associated with a more perturbed HPA axis (2).

IGF1 levels did not differ between T2D and healthy subjects in the present study, suggesting that the GH–IGF1 axis was unaffected in the T2D group. In contrast previous studies have reported low total as well as bioactive IGF1 in subjects with T2D (14, 15). One may speculate that a suppressed GH–IGF1 axis, resulting in low IGF1, occurs first in more advanced metabolic diseases, and had not developed in this group of T2D patients with low HbA1c levels. IGFBP1 levels were similar between the T2D and healthy groups, despite higher insulin levels in T2D, reflecting hepatic insulin resistance (31).

Table 5 Multiple linear regression analyses with peak cortisol after 1 μg ACTH injection as the dependent variable. $n=60$. Disease status healthy = 0, T2D = 1; insulin = basal serum insulin before ACTH injection. Gender categorized as women = 0, men = 1.

Explanatory variables	r^2 of the model	Standardized regression coefficient	*P*
Model 1	0.172		
Gender		0.083	0.083
Disease status		0.221	NS
BMI		−0.235	NS
Waist		0.015	NS
Insulin		0.141	NS
Model 2	0.172		
Gender		0.257	0.045
Disease status		0.223	NS
BMI		−0.244	NS
Insulin		0.143	NS
Model 3	0.128		
Gender		0.258	0.048
BMI		−0.261	0.063
Insulin		0.224	NS

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Limitations in study design may affect the results. OADs may contribute to the differences between groups. Cortisol-binding globulin (CBG) was not measured; however, some studies have shown strong correlations between free (active) and total cortisol, implying no need to correct for CBG (32). CBG is not affected by age, or the menopause (33, 34). Despite its limitations, the present study shows significant differences between patients with T2D and healthy controls, which deserve further study.

In conclusion, men with T2D and good metabolic control had increased adrenal reactivity to ACTH compared with healthy men. This resulted in eradication of the gender difference seen in healthy subjects. IGF1 was unaffected. Further studies will be needed to determine the role of the HPA axis in the pathogenesis of T2D, whether it affects metabolic control and development of complications, and the contribution of pharmacological treatment.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
L Arnetz contributed to data collection and analysis and the writing of the manuscript. N R Ekberg contributed to data analysis and the writing of the manuscript. K Brismar and M Alvarsson contributed to study design and the writing of the manuscript.

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