CLINICAL STUDY

Adipose tissue 11 β -hydroxysteroid dehydrogenase type 1 expression in obesity and Cushing's syndrome

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Abstract

Objective: To evaluate the expression of 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) in omental adipose tissue of patients with Cushing's syndrome and simple obesity, compared with normal weight controls.

Design and methods: We have performed a case-control study and studied omental adipose tissue from a total of 24 subjects (eight obese subjects, ten patients with Cushing's syndrome due to adrenal adenoma, and six normal weight controls). Body mass index, blood pressure, plasma glucose, plasma insulin, plasma cortisol, urinary free cortisol and post dexamethasone plasma cortisol were measured with standard methods. 11 β -HSD1 mRNA and protein expression were evaluated in real-time PCR and western blot analysis respectively.

Results: 11 β -HSD1 mRNA was 13-fold higher in obese subjects compared with controls ($P=0.001$). No differences were found between Cushing's patients and controls. Western blot analysis supported the mRNA expression results.

Conclusions: Our data show the involvement of 11 β -HSD1 enzyme in visceral obesity, which is more evident in severely obese patients than in Cushing's syndrome patients. The lack of increase of 11 β -HSD1 expression in Cushing's syndrome could suggest downregulation of the enzyme as a result of long-term overstimulation.

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Introduction

Intracellular glucocorticoid metabolism is regulated at a prereceptor level (1) by the activity of two isoforms of the 11 β -hydroxysteroid dehydrogenase enzyme: 11 β -Hydroxysteroid dehydrogenase type 1 (11 β -HSD1) is a microsomal enzyme, which acts mainly as a NAD(P)⁺-dependent reductase converting inactive cortisone (E) to active cortisol (F), thus regulating intracellular F access to glucocorticoid receptor (2). This enzyme is widely expressed not only in liver and adipose tissue, but also in adrenal, ovary and decidua (3). 11 β -Hydroxysteroid dehydrogenase type 2 (11 β -HSD2) is a NAD(P)⁺-dependent dehydrogenase, which converts F to E protecting illicit occupation of mineralocorticoid receptor by F in aldosterone target tissues (4). In the obese Zucker rat model, 11 β -HSD1 activity is reduced in liver and increased in omental fat (5, 6), while transgenic mice overexpressing 11 β -HSD1 selectively in adipose tissue develop visceral obesity, dyslipidaemia, insulin-resistant diabetes and hypertension (7, 8). In obese subjects, reduced hepatic 11 β -HSD1 activity has also been described and advocated to activate the hypothalamo–pituitary–adrenal axis (9), while

contrasting data are reported for 11 β -HSD1 expression in fat tissue. Experiments on human adipose tissue have clearly demonstrated that 11 β -HSD1 is expressed both in adipocytes and preadipocytes and its expression and activity are higher in omental than in s.c. preadipocytes (10, 11). In addition, more recently, in stromal cell cultures, a 'switch' in the enzyme activity from dehydrogenase to reductase has been described, despite similar mRNA levels (12). Induction of H6PDH could explain this mechanism across differentiation (13). Relying on the evidence that glucocorticoids induce adipocyte differentiation *in vitro* (11), it has been hypothesized that 11 β -HSD1 dysregulation and an autocrine generation of F in adipocytes may be implicated in the pathogenesis of central obesity. On the other hand, it is well known that F stimulates the 11 β -HSD1 expression and it is possible that higher F levels, as in Cushing's syndrome, can increase the 11 β -HSD1 activity in adipose tissue. In fact, Cushing's syndrome is characterized by abdominal obesity fed by excess glucocorticoid-promoting adipocyte differentiation, so that restoration of normal circulating F levels after adrenal surgery in these patients is associated with a marked reduction of visceral fat.

However, studies on humans have mainly examined s.c. adipose tissue *in vitro* (14–16) and *in vivo* (17) and an increased 11 β -HSD1 expression associated with indices of obesity and metabolic abnormalities has also been shown (18), although 11 β -HSD1 might be more important in omental fat. Many experiments have shown the involvement of peripheral fat in adipose endocrine function, but data supporting the hypothesis that 11 β -HSD1 is increased in omental adipose tissue in human obesity (19, 20) refer to a limited number of studies. To date, no studies have been performed in patients with Cushing's syndrome.

In this work, 11 β -HSD1 gene expression has been evaluated in two different models of omental human adiposity: simple obesity and Cushing's syndrome.

Materials and methods

Human subjects

All patients gave their written informed consent for the collection of adipose tissue and the study was approved by the local ethics committee. Omental tissue was obtained from 24 subjects and periadrenal tissue from three Cushing's patients undergoing abdominal surgery.

For all the subjects, weight, height, body mass index (BMI), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded. A BMI ≤ 25 kg/m² was considered as an index of normality; a BMI > 25 and < 30 kg/m² was considered as an index of overweight and a BMI > 30 kg/m² was considered as an index of obesity. Control subjects had a gender-related body fat distribution. The following biochemical and hormonal parameters were also evaluated: fasting glucose and insulin, plasma F, urinary free F and plasma F after 1 mg overnight dexamethasone suppression test.

Adipose tissue samples were collected from ten patients with full-blown Cushing's syndrome: two with pituitary adrenocorticotrophin (ACTH) secreting adenoma, not cured after pituitary surgery, and eight with hypercortisolism ACTH-independent due to adrenal adenoma, who underwent surgery for bilateral and unilateral adrenal removal respectively. Only the two patients with pituitary ACTH-secreting adenoma were in treatment with ketoconazole (400 mg/day) before adrenal surgery.

Ten omental and three periadrenal adipose tissue samples were collected after adrenal surgery. Eight omental adipose tissue samples were obtained from obese patients undergoing surgery for gastric binding and the remaining omental samples were obtained from six control subjects during colecistectomy. All operations were for non-inflammatory and non-malignant conditions.

For RNA and protein isolation, about 2.0 g whole omental adipose tissue was immediately deep-frozen in liquid nitrogen and stored at -70 °C until extraction.

RNA extraction and reverse transcriptase (RT)

Total RNA extraction was performed with a trizol reaction kit (Invitrogen): 500 mg adipose tissue was homogenized with polytron in 1 ml trizol for each 50 mg tissue. Total RNA was separated from proteins and DNA by adding chloroform and centrifuging at 12 000 *g*, and then recovered from the aqueous phase by precipitation with isopropanol. The RNA concentration was measured by spectroscopy at OD₂₆₀ after the treatment with RNase-Free DNase (Promega). RNA quality was confirmed by electrophoresis on 1% non-denaturing agarose gel.

The RT protocol is as follows: 1 μ g total RNA and 200 ng Random Examers (Applied Biosystems, Foster City, CA, USA) were initially denatured at 70 °C for 10 min. Ten units of multiscribe (RT) (Applied Biosystems), 2.5 mM magnesium chloride, 10 U RNase inhibitor and 0.25 mM each dNTP with 1 $\times$ reaction buffer were added to the RNA and primers in 20 μ l of final volume. The RT reaction was carried out at 42 °C for 50 min and the RT exonuclease activity was then heat-inactivated at 95 °C for 5 min.

Real-time PCR

Real-time PCR was used to make 11 β -HSD1 mRNA quantification. The reaction was carried out using an ABI9700 sequence detection system (Applied Biosystems), which employs TaqMan chemistry for highly accurate quantitation of mRNA levels. Real-time PCR was performed in 25 μ l volumes in a reaction buffer containing TaqMan Universal PCR Master Mix (Applied Biosystems), 3 mM Mn(Oac)₂, 200 μ M dNTPs, 1.25 U AmpliTaq Gold polymerase (Applied Biosystems), 1.25 U AmpErase UNG (Applied Biosystems), 200 nmol TaqMan probe, 900 nmol primers and 25 ng cDNA. All the reactions were performed for the 11 β -HSD1 gene (HSD11B1) and housekeeping gene (18S) at the same time. 18S preoptimized control probe (Applied Biosystems) was used. The data were obtained as Ct values according to the guidelines (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and were used to determine Δ Ct values (Δ Ct = Ct of the target gene – Ct of the housekeeping gene). To calculate the fold changes in gene expression between two categories of patients (e.g. obese and non-obese), the following equation was used; $2^{-\Delta\Delta Ct}$. The measurements were carried out at least three times for each sample.

Primer sequences for HSD11B1 and 18S were obtained from Bujalska *et al.* (12).

Western blot analysis

Western blot analysis for 11 β -HSD1 enzyme was performed in adipose tissue. The samples (0.5 g each) were homogenized in 5 ml buffer containing 25 mM

HEPES, 250 mM sucrose, 4 mM EDTA, 1 μ M leupeptin, 1 μ M aprotinin and 1 μ M pepstatin, pH 7.4. Homogenates were centrifuged at 12 000 *g* for 10 min at 4 °C and the supernatant was collected, aliquoted and stored at –20 °C.

The proteins were measured by the Coomassie Protein Assay Reagent (Pierce) using BSA as a standard. Samples were incubated for 5 min at 95 °C and 20 μ g protein was separated by SDS-PAGE on 10% running gel. The proteins were transferred overnight onto nitrocellulose filters in 20% methanol, 200 mM glycine, 25 mM Tris buffer, pH 8.3. Correct transfer on filters was confirmed by transfer of colour markers (Sigma). The filters were then blocked in 1 $\times$ Tris-buffered saline (TBS) (Tris–HCl and 3 M NaCl) and 5% BSA for 2 h at 42 °C, washed in 1 $\times$ Tween 20 TBS (TTBS) and incubated in 1 $\times$ TBS, 0.5% BSA and 11 β -HSD1 antibody for 2 h at room temperature. Excess antibody was removed by several washes in 1 $\times$ TTBS. Polyclonal antibody against human 11 β -HSD1 raised in sheep (kindly provided by Prof Stewart PM and Bujalska IW) was used at 1:500 dilution. 11 β -HSD1 antibody was tested on human liver and kidney samples, used as control. The liver and kidney were obtained in the course of autopsy after a car accident. The detection of the immunocomplex was performed with ¹²⁵I-labelled G-protein (Amersham) by incubation at room temperature for 45 min. The membranes were thoroughly washed and subjected to autoradiography for 72 h at –70 °C.

Biochemical assays

Insulin levels were determined using an immunoenzymatic assay (AIA-PACK IRI, Eurogenetics Italy, Turin, Italy); the sensitivity of this method is 2.0 μ U/l, intra-assay coefficient of variation (CV) is 4.6% and inter-assay CV is 4.5%.

Plasma glucose was measured by photometric determination using the glucose dehydrogenase method.

Plasma F (5–23 mg/dl, intra- and inter-assay CV values, 9 and 10.3% respectively) was measured by chemiluminescence (Immulite, Diagnostic Products Corporation, Los Angeles, CA, USA).

Urinary-free F was evaluated by RIA (Cortisol Bridge, Athens, Greece; range 7–150 mcg/24 h, intra-assay CV 4.05%).

Statistical analysis

Statistical analysis was performed using the StatView v.4.1 software for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA). Comparisons between groups were performed by ANOVA. Bonferroni's correction was then applied to reduce the probability of differences arising by chance. The data are expressed as mean $\pm$ s.d. Differences were considered statistically significant when *P* was less than 5%.

Results

Clinical and biochemical characteristics of control, obese and Cushing's patients are shown in Table 1.

BMI was significantly lower in Cushing's patients than in obese subjects (*P* < 0.001). DBP values, urinary free F and post dexamethasone plasma F were significantly higher in Cushing's patients than in obese subjects (*P* = 0.045, 0.012 and < 0.001 respectively).

No significant differences were found between Cushing's and obese subjects in SBP, plasma glucose, insulin and plasma F levels (Table 1).

According to ATP III criteria (21), 50% of both Cushing's patients and obese subjects displayed the metabolic syndrome.

Table 1 Clinical and biochemical features of studied patients.

	Patients with Cushing's syndrome (n = 10)	Patients with obesity (n = 8)	<i>P</i> *	Control subjects (n = 6)	<i>P</i> †
Age (years)	45 $\pm$ 15	42 $\pm$ 10	0.676	43 $\pm$ 8	0.879
Sex (M/F)	2/8	3/5	–	2/4	–
Duration of disease (years)	3.5 $\pm$ 2.2	–	–	–	–
BMI (kg/m ²) (range)	29 $\pm$ 3.6 (23–34)	44 $\pm$ 6.0 (35–55)	0.0001	24 $\pm$ 0.8 (23–25)	0.0001
Waist circumference (cm)	97 $\pm$ 12	123 $\pm$ 11	0.0003	77 $\pm$ 8	0.0001
Systolic blood pressure (mmHg)	145 $\pm$ 18	138 $\pm$ 19	0.501	119 $\pm$ 15	0.911
Diastolic blood pressure (mmHg)	94 $\pm$ 7	86 $\pm$ 10	0.045	74 $\pm$ 9	0.003
Plasma glucose (mg/dl)	99 $\pm$ 19	99 $\pm$ 11	0.942	87 $\pm$ 10	0.272
Plasma insulin (mcU/ml)	17.6 $\pm$ 2.2	17.5 $\pm$ 10	0.989	11 $\pm$ 3.5	0.001
HDL cholesterol (mg/dl)	45 $\pm$ 9	43 $\pm$ 8	0.641	52 $\pm$ 5	0.157
Triglycerides (mg/dl)	129 $\pm$ 26	147 $\pm$ 15	0.097	122 $\pm$ 11	0.063
Plasma cortisol, 0800 h (mcg/dl)	23.2 $\pm$ 6.7	16.6 $\pm$ 5.0	0.154	14.7 $\pm$ 4.5	0.021
Urinary free cortisol (mcg/24 h)	431 $\pm$ 374	114 $\pm$ 26	0.012	108 $\pm$ 26	0.001
Postdexamethasone plasma cortisol	17.2 $\pm$ 5.6	1.3 $\pm$ 1.3	0.0001	1.1 $\pm$ 0.7	0.0001

The data are expressed as mean $\pm$ s.d. **t*-Test patients with Cushing's Syndrome versus patients with obesity; †ANOVA test among the three groups of patients.

Adipose tissue expression of 11 β -HSD1

In omental adipose tissue, 11 β -HSD1 mRNA levels were significantly higher in obese subjects compared with both control subjects and Cushing's patients (Δ Ct 13.62 ± 0.15 vs 17.31 ± 0.58 and 17.37 ± 0.68 respectively; $P=0.001$). The mRNA expression was 13-fold higher in obese subjects compared with control subjects, whereas no differences were found between Cushing's patients and controls in omental tissue (Table 2 and Fig. 1). A slight increase was found in periadrenal adipose tissue of Cushing's patients compared with controls (Δ Ct 15.03 ± 0.98 vs 17.31 ± 0.58 ; Table 2).

The mRNA expression data are supported by western blot analysis also, as a higher 11 β -HSD1 protein expression was evident in obese than in Cushing's patients (Fig. 2), but no differences were observed between Cushing's patients and normal weight controls (data not shown).

Western blot analysis in human liver, using a specific 11 β -HSD1 antibody, showed two bands at approximately 34 and 68 kDa confirming the dimeric nature of 11 β -HSD1 in liver. In omental adipose tissue, an additional band of 50 kDa was strongly expressed, so that we found three bands approximately 34, 50 and 68 kDa.

A significant positive correlation between 11 β -HSD1 mRNA expression and BMI was observed analyzing Cushing's patients, obese and control subjects ($r=0.734$; $P<0.001$).

No correlations between 11 β -HSD1 and F, age and gender were found.

Discussion

Excess adipose tissue, mainly localized at the level of the abdomen, is associated with premature mortality from cardiovascular disease. Adipose tissue has been proven to be a real endocrine organ expressing and secreting many factors that act at different levels with both an autocrine/paracrine and endocrine function (22). In a recent study, we highlighted the biological and physiopathological diversities of visceral and s.c. adipose

tissue demonstrating a major role of visceral adipose tissue in determining obesity-related complications (23). Moreover, it has been found that the glucocorticoid action is regulated at a prereceptor level by the activity of 11 β -HSDs, and in particular the 11 β -HSD1 enzyme, which is widely expressed in adipose tissue (3, 10). To our knowledge, this is the first study performed to evaluate the 11 β -HSD1 expression in human visceral adipose tissue of Cushing's patients compared with obese and normal weight controls.

Bujalska *et al.* (10) demonstrated that the 11 β -HSD1 enzyme in omental adipose stromal cells generates active F from inactive E; F generated in this way may act in an autocrine manner on the stromal cells or in a paracrine manner on adjacent adipocytes to promote abdominal obesity. Glucocorticoid receptors found in adipocytes and stromal cells are responsible for adipocyte differentiation, maybe also through the activation of local renin–angiotensin system. The authors suggest that central obesity may reflect 'Cushing's disease of the omentum'. Our data show a significantly higher 11 β -HSD1 mRNA expression in the omental adipose tissue of obese patients compared with control subjects, in accordance with Bujalska *et al.* (10). Our obese patients do not display increased plasma F and/or urinary-free F, supporting the hypothesis that local F produced by increased 11 β -HSD1 isoform in visceral adipose tissue is involved in adipocyte differentiation in an autocrine manner.

Moreover, although evaluated in a small series of samples, the increase of 11 β -HSD1 mRNA expression in the periadrenal adipose tissue of Cushing's patients compared with omental mRNA levels in the same patients suggests a tissue-specific involvement of this enzyme in the adipose tissue of patients with Cushing's syndrome.

Accordingly, with the hypothesis of 11 β -HSD1 tissue-specific involvement, our results are also supported by data from transgenic and knockout mice. Transgenic mice overexpressing HSD11B1 selectively in adipose tissue are obese, hypertensive, dyslipidaemic and insulin-resistant (7), while transgenic mice overexpressing HSD11B1 selectively in liver do not exhibit obesity, but display insulin resistance, fatty liver, dyslipidaemia and hypertension (24). Furthermore, HSD11B1 knockout mice are protected from these metabolic abnormalities (25).

Our data are discordant with those reported by Tomlinson and colleagues (19), who showed the lack of increased 11 β -HSD1 expression in human obesity. However, it is important to underline that these obese patients had BMI much lower than the BMI of our patients (33 vs 44 kg/m²). To this regard, we believe that the different degree of obesity observed between the two groups is determinant for 11 β -HSD1 expression in adipose tissue. The positive correlation of 11 β -HSD1 expression with BMI found in the present study seems to suggest that a marked increase of 11 β -HSD1 is evident only when there is severe obesity and is in accordance

Table 2 11 β -HSD1 expression in human omental and periadrenal adipose tissue by real-time PCR. Values are mean $\pm$ s.d.

	Expression (Δ Ct)	Fold increase versus normal weight controls
Obese subjects (omAT)	13.62 ± 0.1	13
Cushing's patients (omAT)	17.27 ± 1.63	0
Cushing's patients (PAAT)	15.03 ± 0.9	4.9
Normal weight controls (omAT)	17.32 ± 1.1	–

omAT, omental adipose tissue; PAAT, periadrenal adipose tissue.

Figure 1 Vertical point scatterplot comparing HSD11B1 gene expression in normal controls vs obese and Cushing's samples. Five of the six normal controls (83.3%) fell within the normal range (defined as ± 2 s.d. from the mean of the normal controls) compared with none of the obese samples. The difference in the median values between the two groups is statistically significant ($P=0.001$). No differences were found between normal controls and Cushing's samples, because all Cushing's samples fell within normal range. OmAT, omental adipose tissue; PAAT, periaxillary adipose tissue; *vs Cushing's omAT and control omAT; n, number of subjects.

with metabolic variables reflecting insulin sensitivity, as shown by Lindsay *et al.* (26). As for patients with Cushing's syndrome, their BMI is similar to that of the obese patients studied by Tomlinson (19), but again, the data are not comparable because Cushing's syndrome is characterized by hypercortisolism, which is absent in obese subjects, and abdominal adiposity, which is reversible when F levels fall within the normal range, after pharmacological or surgical treatment.

Interestingly, we have found a different expression of 11 β -HSD1 between obese and Cushing's patients. Cushing's patients have a lower 11 β -HSD1 expression in omental tissue compared with obese subjects and their 11 β -HSD1 expression is not different from normal weight controls. The large difference in BMI between obese subjects and Cushing's patients could be one explanation for the different 11 β -HSD1 expression. Cushing's patients develop abdominal obesity, but generally do not display severe obesity as observed in our obese subjects.

Literature data show that F enhances 11 β -HSD1 expression in omental adipose tissue (10, 18), so that in the presence of hypercortisolism, i.e. in Cushing's syndrome, it is licit to expect an increased expression

of this enzyme in such tissue. However, this hypothesis is not confirmed by our results.

The finding of 11 β -HSD1 levels in Cushing's patients that are comparable to those observed in normal weight controls, allow us to reckon that F excess in such patients is not able to induce the expected enzyme upregulation. The lack of increase of 11 β -HSD1 expression in Cushing's syndrome in response to high plasma F levels could suggest a downregulation of the enzyme as a result of long-term overstimulation.

As a possible explanation, we could hypothesize that there is a local defensive mechanism of the adipose tissue in Cushing's patients that prevents 11 β -HSD1 expression, which, in turn, would be responsible for a further F increase.

It would be interesting to evaluate the adipose tissue composition of obese subjects and Cushing's patients also in order to see if a different percentage of preadipocytes and mature adipocytes, as well as adipocyte cell size, could alter the response to F.

Several factors, including some proinflammatory cytokines (27), could regulate and increase 11 β -HSD1 expression in visceral adipose tissue, so that the F produced might be an important autocrine regulator of

Figure 2 Western blot analysis of 11 β -HSD1 in adipose tissue.

fat mass in obese subjects despite normal circulating F levels.

Our expression data are supported by western blot analyses also that showed higher 11 β -HSD1 protein expression in obese subjects. We have found two bands of 34- and 68-kDa in liver; our study therefore confirms not only the dimeric nature of 11 β -HSD1 in liver demonstrated by Maser *et al.* (28), but also highlights the presence of an additional 50-kDa band strongly expressed in omental adipose tissue, consistent with the data reported by Kannisto *et al.* (29). The 50-kDa 11 β -HSD1 protein has not been previously reported in other tissues and neither the nature nor the function of this 11 β -HSD1 subtype is known.

We also observed a significant positive correlation between BMI and 11 β -HSD1 expression analyzing Cushing's patients, obese and control subjects to support a tight correlation between obesity and 11 β -HSD1, in agreement with the previous data on s.c. adipose tissue (14–16). To this regard, the difference in 11 β -HSD1 expression found between obese subjects and all other subjects could be related to the different degree of obesity, which is much more marked in the first group.

The lack of correlation between circulating F levels and 11 β -HSD1 expression in both obese subjects and Cushing's patients, demonstrates that this enzyme is not directly regulated by plasma F concentrations.

In conclusion, the increased expression of 11 β -HSD1 enzyme in omental adipose tissue seems to support its involvement in severe obesity. The higher 11 β -HSD1 expression in obese subjects, compared with control subjects, could sustain that central obesity increases active F from E. The lack of increase of 11 β -HSD1 expression in Cushing's syndrome, in response to high plasma F levels, could suggest a downregulation of the enzyme as a result of long-term over stimulation.

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