Changes in Adenosine 5′-Monophosphate-Activated Protein Kinase as a Mechanism of Visceral Obesity in Cushing's Syndrome

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Objective: Features of the metabolic syndrome such as central obesity with insulin resistance and dyslipidemia are typical signs of Cushing's syndrome and common side effects of prolonged glucocorticoid treatment. AMP-activated protein kinase (AMPK), a key regulatory enzyme of lipid and carbohydrate metabolism as well as appetite, is involved in the development of the deleterious metabolic effects of excess glucocorticoids, but no data are available in humans. In the current study, we demonstrate the effect of high glucocorticoid levels on AMPK activity of human adipose tissue samples from patients with Cushing's syndrome.

Methods: AMPK activity and mRNA expression of genes involved in lipid metabolism were assessed in visceral adipose tissue removed at abdominal surgery of 11 patients with Cushing's syndrome, nine sex-, age-, and weight-matched patients with adrenal incidentalomas, and in visceral adipose tissue from four patients with non-endocrine-related abdominal surgery.

Results: The patients with Cushing's syndrome exhibited a 70% lower AMPK activity in visceral adipose tissue as compared with both incidentalomas and control patients ($P = 0.007$ and $P < 0.001$, respectively). Downstream targets of AMPK fatty acid synthase and phosphoenol-pyruvate carboxykinase were up-regulated in patients with Cushing's syndrome. AMPK activity was inversely correlated with 0900 h serum cortisol and with urinary free cortisol.

Conclusions: Our data suggest that glucocorticoids inhibit AMPK activity in adipose tissue, suggesting a novel mechanism to explain the deposition of visceral adipose tissue and the consequent central obesity observed in patients with iatrogenic or endogenous Cushing's syndrome. **(*J Clin Endocrinol Metab* 93: 4969–4973, 2008)**

Endogenous Cushing's syndrome is caused by either an ACTH-secreting pituitary adenoma, *i.e.* Cushing's disease, or cortisol-secreting adrenal tumors or, more rarely, by ectopic ACTH-secreting tumors. Whereas endogenous Cushing's syndrome is a relatively rare disease with an estimated incidence of five to six cases per 10^6 population per year (1), more than half a million people in the United Kingdom and some 2.5 million people in the United States are currently exposed to long-term glucocorticoid treatments (2–4), resulting in exogenous Cushing's syndrome. Complications such as central obesity, impaired glucose tolerance, dyslipidemia, fatty liver, hypertension, gastritis, osteoporosis and mood alterations contribute to the increased cardiovascular risk and the reduced quality of life, as well as life expectancy, in these patients (5). Central obesity, a typical feature of Cushing's syndrome (6), is characterized by the accumulation of abdominal visceral fat and plays a particularly major role in the development of the metabolic complications.

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Abbreviations: AMPK, AMP-activated protein kinase; FAS, fatty acid synthase; PEPCK, phosphoenol-pyruvate carboxykinase.

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AMP-activated protein kinase (AMPK) is a sensor of cellular energy status (7). AMPK is activated by decreases in the energy state of a cell, and once activated, AMPK switches off anabolic pathways such as fatty acid synthesis as well as protein synthesis and switches on catabolic pathways including glycolysis and fatty acid oxidation.

Many of the changes seen in glucocorticoid excess correspond to metabolic steps regulated by AMPK. In an animal model of Cushing's syndrome, we have recently shown that corticosterone treatment changes AMPK activity in a tissue-specific manner (8): in particular, it causes inhibition of adipose tissue AMPK, which may explain the accumulation of lipids in visceral fat tissue and, together with the abnormal liver AMPK activity, contributes to the development of a fatty liver, dyslipidemia, and insulin resistance. We also observed an increase in hypothalamic AMPK activity in rats in response to glucocorticoid treatment (8), which leads to increased hunger, a known symptom of glucocorticoid excess, even though it is not entirely clear whether this is a direct effect of glucocorticoids or it is due to insulin resistance (9). Data supporting a role for AMPK in the metabolic syndrome have been obtained mainly from studies in rodents, whereas data from humans are limited to studies in skeletal muscle of obese or diabetic patients (10–12). We have now investigated AMPK activity in visceral adipose tissue of patients with Cushing's syndrome.

Patients and Methods

Perirenal visceral adipose tissue was sampled during adrenal operations in 11 patients with Cushing's syndrome (eight patients with cortisol-secreting adenomas, two patients with cortisol-secreting adrenal carcinomas, and one patient with an ACTH-secreting pituitary adenoma (operated for bilateral adrenalectomy because the hypercortisolism was not controlled with the other therapeutic approaches) and nine age-, sex-, and weight-matched control patients with adrenal adenomas that were not associated with symptoms of excess hormone release and were diagnosed as an incidental finding: adrenal incidentalomas. The clinical diagnosis of Cushing's syndrome and the nonhypersecreting adrenal adenomas was made on the basis of the clinical presentation, laboratory testing, and imaging according to published guidelines (5) and was confirmed histologically in all patients. Clinical details of the Cushing's syndrome and the adrenal incidentaloma patients are given in Table 1. An additional control group of visceral adipose tissue from four patients with non-endocrine-related perirenal operations was also analyzed. These patients had no known endocrine disorder but formal testing of the hypothalamus-pituitary-adrenal axis was not performed.

The study was approved by the local Ethics Committee and all patients gave written informed consent to participate in the study. Serum cortisol and ACTH levels were measured by chemiluminescence assays (Diagnostic Products Corp., Los Angeles, CA). Urinary free cortisol was measured by RIA (Cortisol Bridge, Athens, Greece) and HPLC according to the modified Santos-Montes method.

AMPK activity assay

The kinase assay for AMPK activity has been previously described (13, 14). Briefly, samples of adipose tissue were weighed and homogenized with a Precellys 24 machine using CK14 tubes containing ceramic beads (Stretton Scientific, Stretton, UK) at 6000 rpm for one to three cycles of 20 sec in lysis buffer containing phosphatase inhibitors, and the tissue protein content was determined using BCA assay (Pierce, Rockford, IL). AMPK was immunoprecipitated with an equal mixture of α1AMPK and α2AMPK antibodies (13, 14), and AMPK activity was determined by the entity of ^{32}P incorporation into the AMPK substrate SAMS (amino acid sequence: HMRSAMSGLHLVKRR; synthesized by Pepceuticals Ltd., Nottingham, UK). Samples were assayed in duplicate, and each sample was also assayed without the addition of the substrate SAMS as a negative control.

Real-time-PCR

Real-time-PCR using predesigned primers (Applied Biosystems Inc., Warrington, UK) for fatty acid synthase (FAS) and phosphoenol-pyruvate carboxykinase (PEPCK) was performed in human adipose samples following the protocol we previously described (8). The relative quantities of target transcripts were calculated using the standard curve method from the data of triplicate samples after normalization against the housekeeping gene β-actin.

Statistical analysis

Student's *t* test was applied for normally distributed data, whereas the Mann-Whitney *U* test or the Kruskal-Wallis test followed by the Conover-Inman comparison was used for nonnormally distributed data. Correlations were carried out using Spearman's rank correlations. Data are shown as mean ± SEM unless stated otherwise. Symbols used in figures are as follows: *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$, compared with the relevant control.

TABLE 1. Clinical characteristics of the patients

	Cushing's syndrome (n = 11)	Adrenal incidentalomas (n = 9)	*P* value
Age (yr)	50 ± 11	53 ± 8	NS
Sex (M/F)	2/9	5/4	NS
BMI (kg/m^2)	32 ± 7	30 ± 3	NS
WHR	1.01 ± 0.1	0.95 ± 0.1	NS
Adrenal mass size (cm)	4.9 ± 1.8	4.02 ± 0.8	
0900 h cortisol (μg/dl) [nmol/liter]	26.8 ± 7.8 [742 ± 216]	13.8 ± 4.5 [381 ± 124]	<0.001
Urinary free cortisol (μg per 24 h) [nmol per 24 h]	447 ± 564 [1233 ± 1553]	98.1 ± 27.8 [270 ± 76]	<0.0001
Cortisol after Dexa 1 mg (μg/dl) [nmol/liter]	20.5 ± 13.8 [567 ± 383]	2 ± 0.9 [55.5 ± 25]	<0.001
ACTH (pg/ml) [pmol/liter]	6 ± 2.2 [1.3 ± 0.5][a]	27.6 ± 11.5 [6.1 ± 2.5]	0.002

All data are shown as mean ± SD. Normal range for 0900 h serum cortisol is 5–23 μg/dl (138–635 nmol/liter), urinary free cortisol, 13–160 μg per 24 h (35.9–441.4 nmol per 24 h), cortisol after overnight dexamethasone test, 1.8 μg/dl (50 nmol/liter), and ACTH, 7–65 pg/ml (1.54–14.3 pmol/liter). NS, Not significant; WHR, waist to hip ratio; Dexa 1 mg, overnight dexamethasone test with 1 mg oral dexamethasone; BMI, body mass index.

[a] The single patient with pituitary-dependent Cushing's disease with circulating ACTH of 39 pg/ml (8.6 pmol/liter) was not included in the ACTH calculation in the table above.

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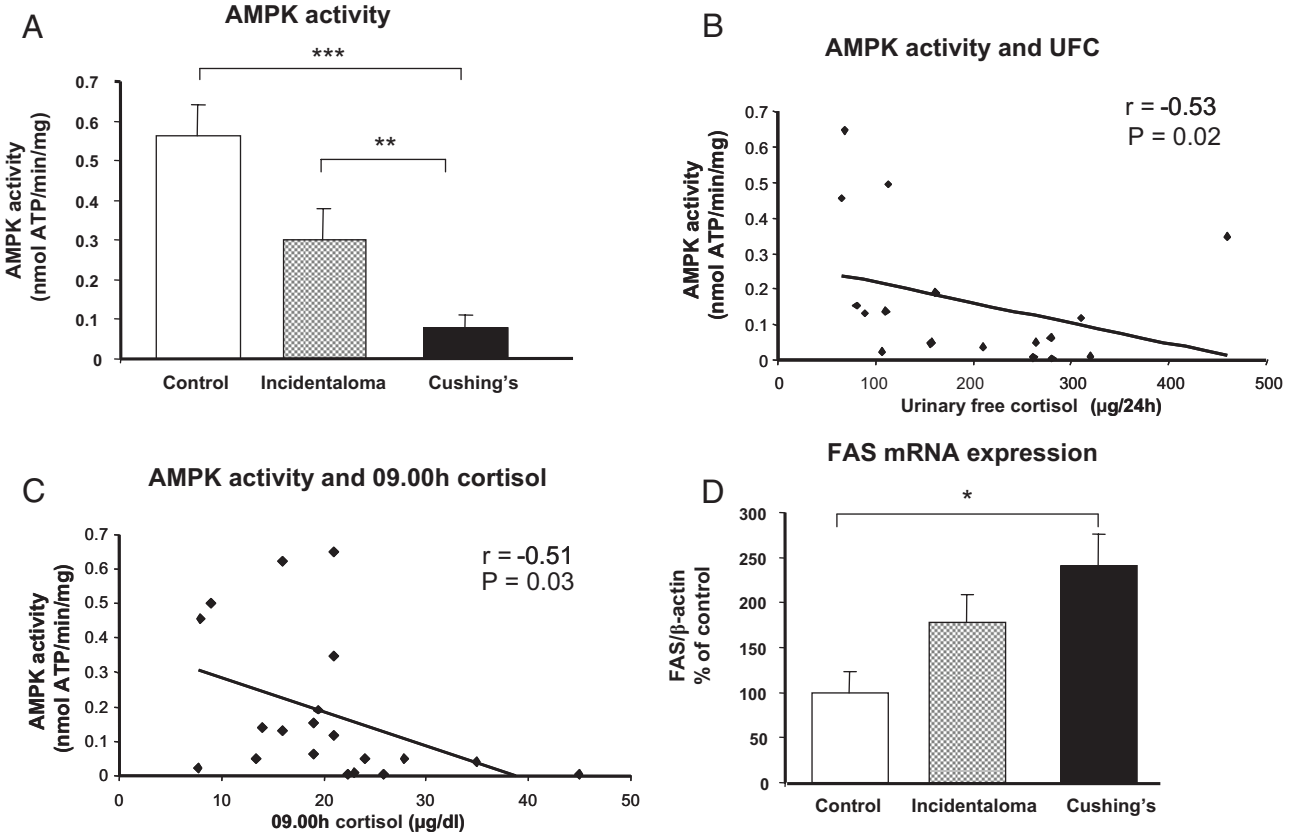

FIG. 1. A, AMPK activity in visceral adipose tissue of patient with Cushing's syndrome compared with patients with nonhypersecreting adrenal adenomas (incidentalomas) and controls. B, Correlation of AMPK activity to urinary free cortisol (UFC) in patients with Cushing's syndrome and adrenal incidentaloma. The patient with exceptionally high UFC of 2215 μg per 24 h was not included in this figure to allow better representation of the data. C, Correlation of AMPK activity to 0900 h serum cortisol in patients with Cushing's syndrome and adrenal incidentaloma. D, FAS mRNA expression in visceral adipose tissue of patients with Cushing's syndrome compared with patients with nonhypersecreting adrenal adenomas (incidentalomas) and controls. Data are shown as mean $\pm$ SEM, n = 4–13 patients/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

Hormone and biochemical measurements in patients with Cushing's syndrome and nonhypersecreting adrenal adenomas (adrenal incidentalomas)

The clinical details of patients with Cushing's syndrome are shown in Table 1. There was a significant difference between patients with Cushing's syndrome and adrenal incidentalomas in the hormonal parameters of cortisol excess: urinary free cortisol ($P < 0.0001$), 0900 h serum cortisol ($P < 0.001$), 0900 h serum cortisol levels after 1 mg dexamethasone at midnight ($P < 0.001$), and plasma ACTH ($P = 0.002$) [the single patient with pituitary-dependent Cushing's disease with circulating ACTH of 39 pg/ml (8.6 pmol/liter) was not included in the ACTH comparison] (Table 1). Some of the patients with adrenal incidentalomas, in accordance with recent clinical data (15–17), had a slight increase in their cortisol burden (subclinical Cushing's syndrome), as demonstrated by the fact that five of nine patients failed to fully suppress their 0900 h serum cortisol to less than 1.8 μg/dl (<50 nmol/liter). Cortisol levels after dexamethasone 1 mg overnight in these five patients were greater than 1.8 μg/dl but less than 5 μg/dl, suggesting that in these patients the more stringent criterion for nonsuppression was not present. The two groups were not significantly different in terms of age, sex, or body mass index.

AMPK activity in visceral adipose tissue

We observed a more than 70% reduction in AMPK activity in the tissue samples from patients with Cushing's syndrome compared with both control groups: AMPK activity was significantly decreased in the visceral adipose tissue samples from patients with Cushing's syndrome compared with patients with adrenal incidentalomas (0.08 ± 0.03 *vs.* 0.3 ± 0.07 nmol/min·mg, $P = 0.007$, Fig. 1A). Because patients with incidentalomas have been shown to occasionally present with signs of mild hypercortisolism (15), we also evaluated AMPK activity in the perirenal adipose tissue from patients with perirenal operations without underlying endocrine disease. The AMPK activity in patients with Cushing's syndrome was again significantly lower compared with these control patients (AMPK activity 0.08 ± 0.03 *vs.* 0.56 ± 0.1 nmol/min·mg, $P < 0.001$, Fig. 1A). The comparison between AMPK activity of adipose tissue from incidentaloma patients and nonendocrine disease patients showed a nonsignificant trend ($P = 0.11$) for lower levels in the incidentaloma patients (Fig. 1A).

In patients with Cushing's syndrome and adrenal incidentalomas, AMPK activity correlated negatively with markers of cortisol burden: urinary free cortisol ($r = -0.53$, $P = 0.02$, Fig. 1B), 0900 h serum cortisol ($r = -0.51$, $P = 0.03$, Fig. 1C), and cortisol levels after 1 mg dexamethasone test ($r = -0.45$, $P = 0.047$).

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RT-PCR

FAS and PEPCK are important rate-limiting enzymes in fatty acid synthesis and glyceroneogenesis. Real-time RT-PCR revealed up-regulation of FAS mRNA in visceral adipose tissue in patients with Cushing's syndrome compared with the control patients (240 ± 34.7% of control, $P = 0.01$, Fig. 1D), whereas values for incidentaloma patients were in between controls and Cushing's patients (178.5 ± 29.9% of control) ($P = 0.12$ *vs.* controls, $P = 0.13$ *vs.* Cushing's). PEPCK mRNA expression seemed to be higher in patients with Cushing's syndrome *vs.* control subjects, but this did not reach significance (228.79 ± 50.5% of control, $P = 0.19$).

Discussion

In states of glucocorticoid excess, there is central obesity with accumulation of the metabolically more disadvantageous intra-abdominal visceral fat (6). Some of the effects of glucocorticoids on adipose cell activity and lipid accumulation are more pronounced in visceral than sc fat, suggesting that glucocorticoids may play a pivotal role in the pathogenesis of the centripetal obesity characteristic of this condition (15, 18). The higher level of the local production of active glucocorticoids from inactive metabolites by visceral adipose tissue 11β-hydroxydehydrogenase-1 in obesity, together with data from the tissue-specific 11β-hydroxydehydrogenase-1 knockout mice, also supports an important role of glucocorticoids in the pathogenesis of visceral adiposity (19). AMPK activation in adipose tissue inhibits lipogenesis and lipolysis and stimulates lipid oxidation (20). Thus, inhibition of AMPK leads to increased lipid stores in association with enhanced lipolysis, leading to the release of free fatty acids (20).

Here we show that excess glucocorticoids are associated with a fall in adipose tissue AMPK activity, and this is supported by our data in a rodent study as well as *in vitro* experiments (8). We had a relatively low number of samples, but it must be considered that endogenous Cushing's syndrome is a relatively rare disease and only a small proportion of these patients undergo abdominal surgery, thereby providing the opportunity to obtain visceral adipose tissue. We used adrenal incidentalomas as a control group for patients undergoing laparoscopic adrenalectomy but also included a second group of patients without any adrenal pathology. Patients with adrenal incidentalomas may show subtle minor defects in corticosteroid secretion and regulation, sometimes resulting in subclinical Cushing's syndrome (15–17). In our cohort five of nine incidentaloma patients did not fully suppress after 1 mg dexamethasone, and indeed the fall in adipose tissue AMPK activity was intermediate in these patients compared with those without any adrenal pathology. Our findings of a decreased AMPK activity in the visceral adipose tissue of patients with Cushing's syndrome with a consequent increase in the expression of the lipid synthesizing enzyme FAS could readily explain the accelerated lipid deposition in visceral adipose tissue upon glucocorticoid excess. Our data also suggest that the suppression of AMPK activity is proportional to the glucocorticoid burden. In agreement with the present results, we previously showed that incubation of human adipocytes with dexamethasone leads to a fall in AMPK activity, indicative of a direct effect of glucocorticoids on human adipocyte AMPK (8). AMPK is a well-known regulator of enzymes in lipid metabolism, and here we show that corticosteroids increase the gene expression of lipogenic enzyme FAS and a trend to higher PEPCK, a known effect of decreased AMPK activity in other tissues (7). However, a direct effect of glucocorticoids via the transactivating effect of the nuclear glucocorticoid receptor may also play a role (21, 22). Patients with Cushing's syndrome have increased visceral but normal or low sc fat, and this corresponds to the data in our animal model of Cushing's syndrome in which glucocorticoids significantly decreased AMPK activity in visceral adipose tissue without an effect on sc adipose tissue in the same animals (8).

Metformin is a mainstay of therapy in the treatment of type 2 diabetes, and its glucose-lowering effects are mediated by liver serine/threonine protein kinase 11 (LKB1), an AMPK upstream kinase (23). Our recent data in human adipocytes show that metformin reverses the inhibitory effects of corticosteroids on AMPK, suggesting that metformin and glucocorticoids influence the AMPK signaling pathway in opposite ways and that metformin is able to override the effect of glucocorticoids on this enzyme. This suggests that metformin or novel tissue-specific AMPK activators could be beneficial in the prevention or treatment of the deleterious metabolic consequences, especially the accumulation of the disadvantageous visceral adipose tissue, in patients with endogenous or iatrogenic Cushing's syndrome. A recognized link between AMPK and cortisol could also improve the development of safer forms of corticosteroid therapy for patients who require the antiinflammatory actions of glucocorticoids because there is an intense search for a glucocorticoid-like compound selectively affecting inflammatory pathways (24); the link suggested by our data between glucocorticoids and AMPK may assist in the selection of the appropriate drug(s) for this purpose.

Acknowledgments

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