Gene expression changes in subcutaneous adipose tissue due to Cushing’s disease

Irit Hochberg¹², Innocence Harvey³, Quynh T Tran⁴, Erin J Stephenson⁵, Ariel L Barkan⁶, Alan R Saltiel², William F Chandler⁶ and Dave Bridges¹³⁷

¹Institute of Endocrinology, Diabetes and Metabolism, Rambam Health Care Campus, Haifa, Israel
²Life Science Institute, University of Michigan, Ann Arbor, MI, USA
³Physiology, UTHSC, Memphis, TN, USA
⁴Preventive Medicine, UTHSC, Memphis, TN, USA
⁵Internal Medicine, University of Michigan, Ann Arbor, MI USA
⁶Neurosurgery, University of Michigan, Ann Arbor, MI USA
⁷Pediatriics, UTHSC, Memphis, TN, USA

Abstract

Glucocorticoids have major effects on adipose tissue metabolism. To study tissue mRNA expression changes induced by chronic elevated endogenous glucocorticoids, we performed RNA sequencing on the subcutaneous adipose tissue from patients with Cushing’s disease (n = 5) compared to patients with nonfunctioning pituitary adenomas (n = 11). We found a higher expression of transcripts involved in several metabolic pathways, including lipogenesis, proteolysis and glucose oxidation as well as a decreased expression of transcripts involved in inflammation and protein synthesis. To further study this in a model system, we subjected mice to dexamethasone treatment for 12 weeks and analyzed their inguinal (subcutaneous) fat pads, which led to similar findings. Additionally, mice treated with dexamethasone showed drastic decreases in lean body mass as well as increased fat mass, further supporting the human transcriptomic data. These data provide insight to transcriptional changes that may be responsible for the comorbidities associated with chronic elevations of glucocorticoids.

Key Words

- Cushing’s syndrome
- lipolysis
- insulin resistance
- glucocorticoid
- lipogenesis
- RNA sequencing
- transcriptome

Introduction

Cushing’s disease, or persistently high circulating levels of cortisol secondary to a pituitary adenoma, leads to significant truncal obesity and diabetes (Cushing 1932). Obesity and diabetes are major factors in morbidity and mortality in Cushing’s disease (Ntali et al. 2015). Cushing’s disease is very rare, with an incidence of 1.2–2.4 per million (Lindholm et al. 2001), but iatrogenic Cushing’s syndrome, caused by chronic glucocorticoid treatment, is very common and leads to similar clinical manifestations.

Numerous studies have shown that glucocorticoids have profound effects on adipose tissue metabolism, including the promotion of adipocyte differentiation (Hauner et al. 1987) and induction of lipolysis and lipogenesis (Divertie et al. 1991, Samra et al. 1998, Kršek et al. 2006, Campbell et al. 2011). Glucocorticoids, through binding to the glucocorticoid receptor, exert transcriptional induction and repression of numerous genes (Reddy et al. 2009, Surjit et al. 2011). Despite the widespread chronic glucocorticoid exposure, there have been no human in vivo studies on global gene expression changes in the adipose tissue in response to long-term exposure to glucocorticoids.
To study the effect of excess endogenous glucocorticoids on adipose tissue, we used RNA sequencing of adipose tissue biopsies from Cushing’s disease patients and controls with non-secreting adenomas. We found a distinctive pattern of changes in many transcripts that are highly associated with Cushing’s disease. Many of these genes explain previously observed metabolic effects of excess glucocorticoids described in vitro, in both animal models and humans. These include enhanced fatty acid and triglyceride biosynthesis, protein degradation, activation of glycolysis and reductions in immune responses.

Materials and methods

Patient recruitment

The study was approved by the institutional review board of the University of Michigan Medical System. Written informed consent was obtained from all of the patients. Patients were recruited consecutively from those undergoing a transsphenoidal adenomectomy at the University of Michigan for Cushing’s disease or nonfunctioning pituitary adenoma over a 12-month period. Exclusion criteria were age <18, current hormone treatment including glucocorticoids, malignancy, inflammatory disease, diabetes type 1 and established pituitary hormone deficiencies. For each patient, a data sheet was completed including, age, sex, anthropometric measurements, diagnosis of hypertension, diabetes, results of blood tests and medications. Fasting blood samples were assayed for glucose (Siemens Advia 1800, Deerfield, IL, USA) and insulin (Life Technologies) as instructed by the manufacturers.

Subcutaneous fat biopsy

During the course of pituitary surgery, a routine subcutaneous fat graft for sealing the surgical field is taken immediately after anesthesia but before glucocorticoid treatment. Approximately 500 mg of this fat graft was used in this study. For ex vivo lipolysis assay, ~100 mg fresh adipose tissue was utilized; ~200 mg was snap frozen in liquid nitrogen and stored at ~80 °C for RNA preparation and ceramide analysis.

Lipolysis assay

Adipose tissue pieces (25 mg) were pre-incubated for 15 min in KRBH buffer (Sigma) at 37 °C and then incubated for 1 h at 37 °C in 300 ml KRBH in duplicate. Glycerol was assayed in supernatants using a glycerol assay kit (Sigma) as instructed by the manufacturer.

Treatment of animals with dexamethasone

Twenty-four C57BL/six adult male mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 9 weeks of age. Following a 1-week acclimation period, mice were either treated with 1 mg/kg per day of dexamethasone (Sigma–Aldrich) in their drinking water (n = 12) or used as controls (n = 12). All animal procedures were approved by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee. Animal body weight and body composition was determined weekly using an echoMRI 2100. Food was weighed weekly, with food intake determined as the decrease in food weight per mouse per week per cage. All mice were provided with access to water ad libitum and a standard rodent diet throughout the study. After 12 weeks of treatment, mice were fasted for 16 h and were sacrificed by cervical dislocation at ZT3 after isoflurane anesthesia. Following cervical dislocation, a sagittal incision was made along the medioventral surface of each mouse and the skin was carefully pulled back to expose the subcutaneous fat depots. The incision was extended along the anterior surface of each hind limb to allow careful dissection of the inguinal fat pads. A small incision was then made into the rectus abdominus muscle to expose the abdominal cavity. The epididymal fat pads were identified and carefully dissected out. The right fat pads from each mouse were weighed and snap frozen in liquid nitrogen for later analysis.

Insulin tolerance test

Insulin tolerance was assessed after 12 weeks of dexamethasone treatment (21 weeks of age). Following a 6-h fast, mice were given i.p. injections of insulin (Humulin R, Lilly, Indianapolis, IN, USA) at a concentration of 1 mU/g. Blood glucose was determined at 15-min intervals post-injection using a One Touch Ultra Glucometer (Lifescan).

Grip test

Grip strength was measured at baseline, 4, 8 and 12 weeks following treatment using a Chatillon digital force gauge (AMETEK, Berwin, PA, USA). Mice were placed on the grid with all four paws in contact with the apparatus and slowly pulled backwards by the tail. Mice were given five trials with about 10 s rests between trials. Grip strength was measured by the average peak torque (n) over the five trials.

Quantitative real-time PCR

RNA was extracted with the PureLink RNA mini kit (Life Technologies). The synthesis of cDNA from 1 µg of RNA
was performed using the High Capacity Reverse Transcription Kit (Life Technologies). cDNA and primers were added to Power SYBR Green PCR Master Mix (Life Technologies) in accordance with the manufacturer’s guidelines and subjected to quantitative real-time PCR as previously described (Lu et al. 2014). The primer sequences used are listed in Table 1. mRNA expression levels of all genes were normalized to ACTB for adipose tissue and GAPDH for muscle tissue after confirming that these mRNAs are unaffected by dexamethasone treatment. Statistical tests were performed as described below based on tests of normality and homoscedasticity, then P values were adjusted for multiple comparisons based on the number of genes tested for each tissue across this manuscript.

Ceramide determination

Ceramide analysis of tissue samples was performed by liquid chromatography-triple quadrupole mass spectrometry according to a modified version of the protocol reported by Kasumov et al. (2010). Briefly, frozen tissue samples were pulverized under liquid nitrogen, then 20 mg portions were extracted using 1.6 ml of a 2:1:0.8 mixture of chloroform:methanol:water containing internal standards (50 ng each of C17 and C25 ceramide and C12 glucosylceramide per sample). The organic layer of the extract was dried under nitrogen gas and reconstituted in 100 μl of 60:40 acetonitrile:isopropanol (Bligh & Dyer 1959). The reconstituted extract was analyzed by electrospray ionization LC-MS/MS on an Agilent (Santa Clara, CA, USA) 6410 triple quadrupole instrument operating in positive ion multiple reaction monitoring mode. The LC column used was a Waters (Milford, MA, USA) Xbridge C18, with 2.5 μm particle diameter, and column dimensions 50 mm (length) × 2.1 mm (inner diameter). Mobile phase A was 5 mM ammonium acetate, adjusted to pH 9.9 with ammonium hydroxide; mobile phase B was 60:40 acetonitrile:isopropanol. The gradient consisted of a linear ramp from 50 to 100%B over 5 min, a 20-min hold at 100%B, and re-equilibration at 50%B for 10 min. The injection volume was 25 μl. Ceramides and glucosylceramides were identified by retention time and by MS/MS fragmentation parameters and were quantitated by peak area relative to the closest matching internal standard using Agilent MassHunter Quantitative Analysis software.

Transcriptomic analysis

Total RNA was extracted from adipose tissue using the RNEasy kit (Qiagen) and its quality was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies). At the University of Michigan DNA Sequencing Core, cDNA libraries from polyA mRNA were prepared using a TruSeq cDNA synthesis kit and sequenced using a HiSeq 2000

**Table 1** Primer sequences used for qPCR analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acaca</td>
<td>GCTAAAACCGACCTCCCGAT</td>
<td>GTATCTGAGCTGACGGAGGC</td>
</tr>
<tr>
<td>Aco1</td>
<td>GGCATTGGCCTCCCTTCTTGCA</td>
<td>GTATGAGCTGACGGAGGC</td>
</tr>
<tr>
<td>Acsl1</td>
<td>GCTGCTGGGAGAAGCCAC</td>
<td>GGCAAAGTCATCTGACGCT</td>
</tr>
<tr>
<td>Acs2</td>
<td>ATGTGATCAGCCAGAGCAGG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Acb</td>
<td>ATGTGATCAGCCAGAGCAGG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Agpat2</td>
<td>AACTGCCAAGAAGCTGAGG</td>
<td>GGAAAGTCTCGCAGTCG</td>
</tr>
<tr>
<td>Dgat2</td>
<td>ATGGCTGACCTCACTCGTG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Dhr7</td>
<td>AGTTCGCACTCCCTCCTTG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Dhr24</td>
<td>AGTTCGCACTCCCTCCTTG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Fasn</td>
<td>GGAGTTGTGATAGCCGAGCAA</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Fbox32</td>
<td>CTTCGCACTCCCTCCTTG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Gapdh</td>
<td>GCAATGCTCTCTGCTATCCATG</td>
<td>GGTGACACACTCCCTTCTCCAG</td>
</tr>
<tr>
<td>Gpam</td>
<td>GTGGAAGGAGATGATAGGACCA</td>
<td>GGAAAGTCTCGCAGTCG</td>
</tr>
<tr>
<td>Gpd1</td>
<td>TCTCGACCTCACTCGTG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Idh1</td>
<td>ATGTGATCAGCCAGAGCAGG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Ldhb</td>
<td>ATGTGATCAGCCAGAGCAGG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Mdh1</td>
<td>AACTGCCAAGAAGCTGAGG</td>
<td>GGAAAGTCTCGCAGTCG</td>
</tr>
<tr>
<td>Me1</td>
<td>GACCGCCATCTCAACAGA</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Psmd1</td>
<td>TGGTCACTCATGGGTAGGACA</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Psmd8</td>
<td>AGCAGGCTCAGCCATCGTG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Riplp0</td>
<td>GAACTGCTGCTCCTCCTCCG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Riplp13a</td>
<td>GGAACTGCTGCTCCTCCTCCG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Scd1</td>
<td>ACCTGCCCTACCAACAGCG</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
<tr>
<td>Trim63</td>
<td>GAGGGCCATTGACTTTGGGA</td>
<td>GGGAGATGGGAGACCAC</td>
</tr>
</tbody>
</table>
Table 2 Clinical characteristics of Cushing’s disease and control patients. Data represents mean ± s.e.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cushing’s disease (n=5)</th>
<th>Controls (n=11)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td>166 ± 4.3</td>
<td>169 ± 2.4</td>
<td>0.47</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>91.9 ± 9.1</td>
<td>89.6 ± 6.7</td>
<td>0.89</td>
</tr>
<tr>
<td>BMI</td>
<td>33.3 ± 3.8</td>
<td>30 ± 1.8</td>
<td>0.52</td>
</tr>
<tr>
<td>Abdominal circumference (cm)</td>
<td>112.4 ± 6.4</td>
<td>100.65 ± 4.4</td>
<td>0.16</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>0.95 ± 0.3</td>
<td>1.96 ± 0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39.8 ± 4.5</td>
<td>63.4 ± 2.7</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

(Illumina, San Diego, CA, USA). Samples were run on two lanes of a HiSeq 2000 (Illumina) generating 8 612 682–16 469 501 single-ended 50 bp reads per sample. These were aligned to the human genome (Ensembl GRCh37.74, Genbank Assembly ID GCA_000001405.14) using the TopHat version 2.0.10 (Kim et al. 2013), Bowtie 2 version 2.1.0 (Langmead & Salzberg 2012) and Samtools version 0.1.18. Reads were mapped to known genes using Htseq (Anders et al. 2015). Gene expression was analyzed using DESeq2 version 1.2.10 (Love et al. 2014). The analysis we focused on in this manuscript was without adjustment for BMI or age and is presented in Supplementary Table 2, with the exception of subjects 29 and 31 (both Cushing’s disease patients), who had clinical data but no RNAseq data.

Statistical analysis

Descriptive statistics including means and s.e.s were determined for clinical measurements. All of the statistical tests were performed using the R package (version 3.0.2 (R Core Team 2013)). Normality assumption was checked via the Shapiro–Wilk test. Wilcoxon rank sum tests were used when data were not normally distributed. Welch’s t-test was performed if the equal variance assumption was rejected by Levene’s test (car package version 2.0-19), otherwise a Student’s t-test was used. Longitudinal measurements such as body weight, food intake, body composition and insulin tolerance tests were analyzed via mixed linear models and a χ² test between models with and without dexamethasone treatment as a covariate. This used the lme4 package, version 1.1-7 (Bates et al. 2014).

Table 3 Summarized gene set enrichment analysis of pathways. Selected pathway enriched in subcutaneous adipose tissue from Cushing’s disease patients via GSEA analysis. For a complete list, see Supplementary Tables 2 and 3

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Dataset</th>
<th>NES</th>
</tr>
</thead>
<tbody>
<tr>
<td>M_PHASE_OF_MITOTIC_CELL_CYCLE</td>
<td>Gene ontology</td>
<td>2.60*</td>
</tr>
<tr>
<td>KEGG_CITRATE_CYCLE_TCA_CYCLE</td>
<td>KEGG</td>
<td>2.41*</td>
</tr>
<tr>
<td>KEGG_BIOSYNTHESIS_OF_UNSATURATED_FATTY_ACIDS</td>
<td>KEGG</td>
<td>2.41*</td>
</tr>
<tr>
<td>REACTOME_TRIGLYCERIDE_BIOSYNTHESIS</td>
<td>Reactome</td>
<td>2.24*</td>
</tr>
<tr>
<td>PYRUVATE_METABOLISM</td>
<td>Gene ontology</td>
<td>2.24*</td>
</tr>
<tr>
<td>KEGG_VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION</td>
<td>KEGG</td>
<td>2.16*</td>
</tr>
<tr>
<td>STEROID_BIOSYNTHETIC_PROCESS</td>
<td>Gene ontology</td>
<td>2.11*</td>
</tr>
<tr>
<td>KEGG_STARCH_AND_SUCROSE_METABOLISM</td>
<td>KEGG</td>
<td>2.08*</td>
</tr>
<tr>
<td>PROTEASOME_COMPLEX</td>
<td>Gene ontology</td>
<td>1.78*</td>
</tr>
<tr>
<td>KEGG_ALLOGRAFT_REJECTION</td>
<td>KEGG</td>
<td>1.87*</td>
</tr>
<tr>
<td>KEGG_BASAL_CELL_CARCINOMA</td>
<td>KEGG</td>
<td>1.86*</td>
</tr>
<tr>
<td>KEGG_RIBOSOME</td>
<td>KEGG</td>
<td>2.33*</td>
</tr>
</tbody>
</table>

NES, net enrichment score; *q < 0.25.
study are available through the Gene Expression Omnibus (GSE66446) and at http://bridgeslab.github.io/Cushing AcromegalyStudy (Bridges & Tran 2015).

Results

Patient characteristics

Clinical and metabolic measurements were obtained for five Cushing’s disease patients and 11 control subjects, who were admitted with non-secreting adenomas. Patient characteristics are shown in Table 2. Our Cushing’s disease patients were, in general, younger and had smaller tumors than the patients with non-secreting adenomas. In the Cushing’s disease cohort there was a nonsignificant elevation in body weight (P = 0.47), BMI (P = 0.27) and abdominal circumference (P = 0.07, Fig. 1A), consistent with Cushing’s disease patients with elevated fat mass and truncal obesity (Lamberts & Birkenhæger 1976).

We detected a nonsignificant elevation in the HOMA-IR score (2.6-fold, P = 0.67 by Wilcoxon test, Fig. 1B), driven largely by increases in fasting insulin levels (P = 0.30). Three out of the five Cushing’s disease patients had diabetes, while only one of the 11 controls had diabetes (P = 0.03 via χ² test). These data are consistent with elevated glucose intolerance in patients with Cushing’s syndrome. We observed significant elevations in both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum from Cushing’s disease patients. To evaluate lipolysis in explants from these patients, we measured glycerol release from isolated subcutaneous adipose tissue in explants from these patients. To evaluate insulin sensitivity, we performed insulin tolerance tests on these mice after 12 weeks of dexamethasone treatment and found that while they had reduced fasting glucose at this stage, they were resistant to insulin-induced reductions in blood glucose (Fig. 2E). On sacrifice after 12 weeks of dexamethasone treatment, the adipose tissue was dissected and weighed. As shown in Fig. 2F, we observed elevated subcutaneous fat mass in dexamethasone-treated animals.

Transcriptomic analysis of human adipose tissue from Cushing’s patients

To determine which genes and pathways are altered in the adipose tissue in the human Cushing’s disease subjects,
we analyzed the transcriptome from subcutaneous adipose tissue mRNA from the five Cushing’s disease patients and 11 controls. We identified 473 genes that had significantly different expressions in Cushing’s disease patients; of these, 192 genes were expressed at a lower level and 281 at a higher level in the adipose tissue from the disease patients. These transcripts form a signature identifying transcriptional difference in the adipose tissue in response to long-term exposure to glucocorticoids (Fig. 3A).

To identify conserved pathways underlying these changes, GSEA was performed on these data. As summarized in Table 3, we detected the enrichment of genes in several categories involved in metabolism, including a higher expression of gene sets involved in lipid biosynthesis, glucose metabolism, activation of amino acid degradation, protein degradation and reductions in protein synthesis. We also observed reduced expression of transcripts involved in immune function. These will be discussed in subsequent sections.

We next evaluated the levels of the glucocorticoid receptor (NR3C1) and the mineralocorticoid receptor (NR3C2) and observed no significant downregulation of following a 6-h fast, insulin (1 mU/g) was administered via i.p. injection, and blood glucose was measured at baseline and the indicated time post injection. (G) Inguinal (IWAT) and epididymal (EWAT) fat pad weights, for right fat pads only. *P<0.05.
these receptors at the mRNA level in Cushing’s patients (Fig. 3B). Another potential mechanism for negative feedback of glucocorticoid signaling is through the enzymatic activities of 11β-HSD1/2, which control the local concentrations of cortisol in adipose tissues. We observed a nonsignificant reduction in HSD11B1 mRNA levels (24% reduced, P value adjusted (Padj) = 0.49), potentially desensitizing adipose tissue to cortisol by reducing the conversion of cortisone to cortisol. The induction of leptin by glucocorticoids has been previously reported in human adipocytes (Halleux et al. 1998) and in human adipose tissue in vivo (Papaspyprou-Rao et al. 1997). We observed a 1.8-fold higher level of leptin (LEP) expression and nonsignificantly higher resistin (RETN) expression but no significant changes in adiponectin mRNA levels (ADIPOQ, Padj = 0.94; Fig. 3C).

**Lipogenesis genes are upregulated in response to elevated glucocorticoids**

Increased subcutaneous fat mass is a hallmark of Cushing’s syndrome and could potentially be mediated through the activation of adipogenesis or lipogenesis. Our transcriptomic data support the hypothesis that lipogenesis is activated in these tissues via the transcriptional activation of fatty acid synthesis and triglyceride synthesis. All of the major genes involved in the synthesis of fatty acids were expressed at higher levels including ACACA, FASN, ACSL1/3/4 and ELOVL1/5/6 (Fig. 4A). Desaturation of fatty acids is an essential aspect of de novo fatty acid synthesis, and we also observed elevations in all fatty acid desaturases SCD, FADS1, FADS2 and HSD17B12 (Fig. 4B). The triglyceride synthesis genes GPAM, DGAT1, DGAT2, AGPAT2/3 and GPDI were also upregulated in the subcutaneous adipose tissue from Cushing’s disease patients (Fig. 4C).

In spite of the increased lipid deposition and elevations of lipogenesis genes in Cushing’s disease patients’ adipose tissue, there have been several studies linking elevated glucocorticoids to increased lipolysis. In our patients, this was observed in ex vivo explants of subcutaneous adipose tissue (Fig. 1D). Among genes that may liberate fatty acids from triglycerides, lipoprotein lipase (LPL) was induced 1.45-fold (Padj = 0.055) in the Cushing’s disease subjects, but neither hormone sensitive lipase (LIPE) nor adipose triglyceride lipase (PNPLA2) were significantly changed at the transcriptional level (Fig. 4D). It is possible that insulin resistance due to glucocorticoids caused decreased repression of lipolysis leading to its upregulation. However, our data supports an insulin-independent activation as well, because in our explants, insulin was not present during the lipolysis assay. We detected an elevation of perilipin 4 (PLIN4), which is one of the proteins that coat intracellular lipid storage droplets (induced 1.45-fold, Padj = 0.056; Supplementary Table 1).

Several genes that regulate steroid biogenesis were elevated in the adipose tissue from Cushing’s disease patients. Among these, a significant increase was observed for the P450 oxidoreductase (POR) mRNA (87% increase, Padj = 0.05; Fig. 4F). This enzyme is involved in the production of androgenic hormones, and its upregulation is consistent with the increased levels of androgens observed in Cushing’s disease. The expression of the androgen receptor (AR) was also significantly increased (55% increase, Padj = 0.05; Fig. 4F), indicating an increased sensitivity to androgens in these patients. These findings suggest that glucocorticoids may play a role in the increased expression of androgenic genes in Cushing’s disease, potentially contributing to the development of androgenic features observed in these patients.
patients as described in Fig. 4E. These include several cytochrome P450 family members, steroid reductases (SRD5A1, SRD5A3), Aldo-keto reductase family 1 member C1 (AKR1C1), 7-dehydrocholesterol reductase (DHCR7) and HMG-CoA synthase (HMGCS1).

To examine whether lipogenesis genes are activated in the dexamethasone-treated mice, we tested several of these genes in the subcutaneous adipose tissue from dexamethasone-treated mice and observed elevations in FASN, GPAM, GPD1, ACS2, ACS1, DGAT, AGPAT2, DHCR7/24 and ACACA1 (Fig. 4F). In contrast to the human samples, we did not observe an elevation in the mouse isoform of SCD but did see instead a reduction in Scd1 mRNA.

**Genes controlling glucose oxidation are elevated in Cushing’s disease patients**

Several glucose metabolism genes, and specifically glycolysis and tricyclic acid (TCA) cycle genes were expressed at higher levels in Cushing’s disease patients (Fig. 5). Strongly induced genes included HK3, FBP1, ALDOC, ENO1, IDH1, ME1 and DLAT. Upregulations in IDH1 and ME1 were also noted in the mouse adipose tissue, along with other transcripts involved in glucose oxidation such as ACO1, LDHB and MDH1 (Fig. 5C).

The major glycogen synthesis transcripts were also induced, including GYS2, UGP2 and GBE1. This agrees with biochemical studies that implicate glucocorticoid treatment in elevated hepatic and adipose tissue glycogenesis (Engel & Scott 1951, Segal & Gonzalez Lopez 1963, Baqué et al. 1996). The relevance of this effect in adipose tissue has not yet been explored.

**Genes that regulate protein catabolism are upregulated in adipose tissue from glucocorticoid exposed subjects**

We found that two major pathways of protein homeostasis are altered in response to glucocorticoids. In concert with reductions in lean body (including muscle) mass (Fig. 2B), we observed substantial muscle weakness in mice treated with dexamethasone (Fig. 6A). In a separate cohort of mice, after 1 week of dexamethasone treatment in skeletal muscle, mRNA levels of the E3 ligases (Atrogin-1 and

---

**Figure 5**

Glycolysis and glucose oxidation genes are upregulated with elevated glucocorticoids. Schematic of (A) glycolysis and (B) the TCA cycle, colored by gene expression changes in subcutaneous adipose tissue from Cushing’s disease subjects. (C) qPCR analysis of selected glucose oxidation genes from mouse subcutaneous adipose tissue after 12 weeks of dexamethasone treatment. *p<0.05.
MuRF1) were induced as was the proteasomal gene PSMD8 (Fig. 6B). These effects did not reach statistical significance due to variability in dexamethasone responsiveness. Similar inductions of the proteasomal genes were observed in the subcutaneous adipose tissue from the cohort of mice treated with dexamethasone for 12 weeks (Fig. 6C).

In the adipose tissue from Cushing’s disease patients, we observed inductions of both the proteasomal pathways (via KEGG, net enrichment score 1.76, Padj = 0.01; Fig. 6D) and genes involved in amino acid catabolism (Fig. 6E) and a general downregulation of ribosomal genes (Fig. 6F). Among the amino acid catabolism genes, AOX1 (96% increase, Padj = 0.03), OXCT1 (40% increase, Padj = 0.04) and BCAAT1 (80% increase, P = 0.048) were all significantly upregulated. Together these data support the hypothesis that protein catabolism and a reduction in protein synthesis also occur in adipose tissue in response to glucocorticoid exposure.

Genes involved in proximal insulin signaling are unchanged in adipose tissue from Cushing’s disease patients

As described in Figs 1B and 2F, we observed insulin resistance in concert with elevated glucocorticoid levels in both mice and humans. Several mechanisms have been proposed linking glucocorticoids to insulin sensitivity including elevated lipolysis. As shown in Fig. 7A, there was a slightly higher expression of insulin pathway transcripts including FOXP1, the insulin receptor (INSR), the insulin receptor substrates IRS1 or IRS2 and the p85 regulatory subunit of phosphoinositide-3-kinase (PIK3R1), consistent with previous studies (Gathercole et al. 2007, Tomlinson et al. 2010, Hazlehurst et al. 2013), though in our hands none of these genes reached statistical significance. The insulin pathway was generally expressed at significantly higher levels in the Cushing’s disease patients compared to controls (KEGG pathway, net enrichment score 1.84, Padj = 0.006). These data do not support transcriptional downregulation of proximal insulin signaling genes as mediating insulin resistance in subcutaneous adipose tissue.

Changes in cell ceramide and glucosyler ceramide have been suggested to be important in vitro and in obesity- and glucocorticoid-induced insulin resistance in skeletal muscle (Adams et al. 2004, Aerts et al. 2007, Holland et al. 2007). To test biochemically whether ceramides may play a role in the Cushing’s disease-associated insulin resistance, we treated with dexamethasone for 12 weeks. Proteasomal (D) and protein catabolism (E) transcript expression changes in subcutaneous adipose tissue from Cushing’s disease and control subjects. (F) Heatmap of differentially expressed ribosomal transcripts in Cushing’s disease and control subjects. * indicates q < 0.05.

Figure 6

Increased glucocorticoids are associated with increased protein degradation and decreased strength. (A) Mouse grip strength (N) assessed at baseline, 4, 8 and 12 weeks of dexamethasone treatment. Muscle atrogene (B) and proteasomal transcript expression changes in gastrocnemius muscles from mice following 1 week of dexamethasone treatment. (C) Proteasomal mRNA levels from subcutaneous adipose tissue of mice treated with dexamethasone for 12 weeks. Proteasomal (D) and protein catabolism (E) transcript expression changes in subcutaneous adipose tissue from Cushing’s disease and control subjects. (F) Heatmap of differentially expressed ribosomal transcripts in Cushing’s disease and control subjects. * indicates q < 0.05.
resistance in adipose tissue, we took a lipidomics approach to analyze the ceramide species from the adipose tissue explants of the same patients. We observed no statistically significant changes in any ceramide species (Fig. 7B, q > 0.25).

Inflammation

Several pathways involved in immune function were downregulated in the adipose tissue from Cushing’s disease patients. This is consistent with the effects of cortisol in suppressing immune function generally. Adipose tissue leukocyte infiltration both relies on an intact immune system and responds to changes in adiposity (Lumeng & Saltiel 2011). Among immune genes, we detected reductions in several genes that form the class II major histocompatibility complex, notably HLA-DPB2, HLA-DRA, HLA-DRB9 and HLA-DQA1 (Fig. 7C). These genes normally present antigens for T-cell recruitment. Consistent with this, we observed reductions in the mRNA of IL32, a hormone secreted by natural killer and T lymphocytes (Dinarello & Kim 2006). We also observed a downregulation in transcripts that are interferon-gamma dependent. Together, these data support the hypothesis that the decreased T-cell activation observed with cortisol signaling also impacts adipose tissue.

Modifying effect of obesity on glucocorticoid responsiveness

In our small cohort of Cushing’s disease subjects, we examined whether some of the dramatic transcriptional changes were modified by the obesity status of the patients (based on a BMI cutoff of 30; Supplementary Tables 4 and 5). We were surprised to note that many genes that had strongly elevated transcripts in non-obese Cushing’s disease patients had blunted effects in obese Cushing’s disease patients. Some examples of this include FASN, PSMD8 and IDH8 (Fig. 8A, B and C). Among genes that were more strongly induced in obese patients, most of these are involved in lysosomal function, including the cathepsins (CTSB, CTSD, CTSZ), LAPTMS and LIPA (Fig. 8D). Although the small number of obese and non-obese Cushing’s patients in our study makes these provocative observations, preliminary, it is suggestive of both a general reduction of glucocorticoid sensitivity in obese subjects and potentially an underappreciated role of lysosomes in obese patients with elevated cortisol levels.

Discussion

In this study we have described a transcriptional signature in the adipose tissue from subjects with Cushing’s disease and verified several of these changes using a mouse model of glucocorticoid treatment. We have identified several pathways that are significantly changed in response to chronic glucocorticoid exposure. Broadly, these changes reflect a shift toward more a rapid metabolism of glucose through glycolysis and the TCA cycle and a shifting of glucose and protein metabolites toward lipogenic pathways in adipose tissue. This is indicated by significant increases in glycolytic (ALDOC, ENO1, IDH1, ME1, G4MAL and GAPDH), proteolytic (PSMD1/14) and lipogenic (ACACA, FASN, ACSL1, ELOVL5, GPAM and DGAT2) transcripts in human adipose tissue, with similar transcript
Transcript expression changes in Cushing’s disease are less robust after adjusting for obesity. FASN (A), PSMD8 (B), IDH1 (C), and lysosomal (D) transcripts in non-obese and obese Cushing’s subjects. * indicates q < 0.05.
There are several limitations to our evaluation of insulin sensitivity in this study. One aspect is that two of the three patients with Cushing's syndrome and diabetes were treated with antidiabetic medications. Secondly, it is possible that insulin resistance in these subjects/mice is mainly due to muscle or liver insulin resistance and that adipose tissue may respond to insulin in a relatively normal fashion. Glucocorticoid-induced insulin resistance is thought to be mostly secondary to the increase in free fatty acids caused by the induction of lipolysis (Geet et al. 2014). Results from a recent study imply that glucocorticoids do not induce resistance in subcutaneous adipose tissue in vivo in healthy subjects (Hazelhurst et al. 2013), suggesting that peripheral insulin resistance may not occur in adipocytes and that whole-body insulin resistance may primarily occur in muscle and liver tissues. This is consistent with our observations of a lack of changes in proximal insulin resistance in muscle and liver tissues. This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-15-0119.

Another limitation in our study is the small sample size, especially the number of biological replicates in the Cushing's group \((n = 5)\). Adding a covariate such as BMI or age in the model further reduces the sample size to two or three replicates. Although this sample size is small, it is reasonable for a rare disease such as Cushing's. Realizing our limitation, we chose DESeq2 as the statistical method for our RNAseq data. DESeq2 overcomes the small number of biological replicates in the maximum group. A empirical Bayes approach is then used to get maximum a posteriori as the final dispersion estimate. This method utilizes the available data to the maximum extent and, therefore, helps avoid potential false positives (Love et al. 2014).

These data provide a variety of novel transcriptional changes that may be causative of the comorbidities associated with Cushing's disease. Further studies in animals and cells using knockout or overexpression of specific transcripts may verify which of the changes is crucial in the metabolic effects of glucocorticoid effects in adipose tissue.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-15-0119.

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.

Funding
This work was supported by Motor City Golf Classic (MCGC) Grant number G010640 and Le Bonheur Grant number 650700. This work utilized Metabolomics Core Services supported by grant U24 DK097153 of the NIH Common Fund to the University of Michigan.

Author contribution statement
I Ho conceived the study, and D B, A R S and I Ho provided funding. W F C and A L B recruited the patients and obtained clinical data. W F C supplied the biopsies and serum samples. I Ho assayed the tissues for lipolysis and performed the serum measurements. Q T, D B, I Ha and I Ho analyzed the RNAseq data. I Ha generated the mouse data with assistance from E J S. This was analyzed by I Ha, D B and Q T. I Ho and D B wrote the manuscript.

Acknowledgements
We thank Charlotte Gunden, Elizabeth Walkowiak and Eric Vasbinder for their valuable help in the study. The authors would like to thank Ian Brooks and the UTHSC-ORNl Center for Biomedical Informatics for provisioning the RStudio server used in this analysis. We would also like to thank the University of Tennessee Health Science Center Neuroscience Institute for use of the grip strength monitor. We would also like to thank the Molecular Resource Center at the University of Tennessee Health Science Center for qPCR facilities.

References


DNA analysis of adipose from Cushing’s disease


Lumeng CN & Saltiel AR 2011 Inflammatory links between obesity and metabolic disease. Journal of Clinical Investigation 121 2111–2117. (doi:10.1172/JCI57142)


Segal HL & Gonzalez Lopez C 1963 Early effects of glucocorticoids on precursor incorporation into glycogen. *Nature* **200** 143–144. (doi:10.1038/200143a0)


Received in final form 26 June 2015
Accepted 6 July 2015
Accepted Preprint published online 6 July 2015