Chapter 11

Glucocorticoids alter Subcutaneous Adipose Tissue Function and Adipokine Levels in vivo in Healthy Men

ABSTRACT

Introduction: Glucocorticoid (GC) treatment is associated with diabetogenic side effects. However, the effects on adipose tissue function are currently not completely clear. Evidence for insulin resistance and increased lipolysis was observed in vitro. In the present study we assessed the effects of low- and high-dose prednisolone treatment on adipose tissue function and circulating levels of adipokines in vivo in healthy men.

Research design and methods: Healthy men (n=24; age 21.1±2.1 years; BMI 21.9±1.7 kg/m²) were allocated to a two-week treatment with prednisolone 7.5 mg or 30 mg daily for two weeks. At baseline and on day 14 of treatment, insulin sensitivity was measured by hyperinsulinemic-euglycemic clamp and plasma levels of adiponectin, resistin and leptin were determined. An abdominal subcutaneous adipose tissue (SAT) biopsy was taken in the fasted state and during insulin infusion before and after the intervention to study insulin signaling (protein kinase B (PKB/Akt) and proline-rich Akt substrate (PRAS)-40 phosphorylation). In addition, adipose tissue protein expression of adipogenesis markers (peroxisome-proliferator activated receptor (PPAR)-γ and Kruppel-like factor (KLF)-4), lipolytic enzymes (adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL)) and adiponectin gene expression was measured. Treatment-induced changes were compared to baseline using paired statistical tests.

Results: Prednisolone 30 mg increased fasting glucose levels (P<0.01) and decreased whole-body insulin sensitivity (P<0.01). Furthermore, prednisolone 30 mg reduced PKB/Akt phosphorylation in SAT, and reduced the expression of ATGL (all P<0.05). Moreover, prednisolone 30 mg reduced PPAR-γ and KLF-4 expression (P<0.05). Finally, prednisolone 30 mg increased resistin (P=0.02) and leptin levels (P=0.05) while a trend towards plasma adiponectin lowering was observed (P=0.07). Prednisolone 7.5 mg treatment decreased PPAR-γ, KLF-4 and ATGL expression (all P<0.05) in the absence of other effects.

Conclusions: High-dose prednisolone treatment negatively affected SAT function by interfering with insulin signaling and decreasing the expression of adipogenesis markers, which could result in decreased SAT mass and untoward adipose tissue distribution. In addition, circulating adipokine profile was unfavorably changed by high-dose prednisolone. Low-dose prednisolone treatment impaired markers of adipogenesis. Treatment-related changes in SAT function and circulating levels of adipokines may be among the mechanisms mediating diabetogenic effects of GC treatment.
Glucocorticoids (GCs) are important regulators of metabolic homeostasis, but have diabetogenic effects, in particular when present in supraphysiological concentrations [1]. Although the adverse metabolic effects of elevated GC levels on glucose [2] and protein metabolism [3] are reasonably well defined, the effects on lipid metabolism are less clear [4]. In addition, the involvement of a number of organs in the development of the diabetogenic effects of GC treatment, including pancreatic islet cells, skeletal muscle and liver are understood to a greater extent than the role of adipose tissue. However, several observations indicate that GCs may exert untoward effects on adipose tissue.

As such, chronic GC excess in Cushing’s syndrome or during GC treatment induces fat deposition in the visceral compartment [5] and promotes liver fat accumulation [6], with wasting of subcutaneous adipose tissue depots. This increment in visceral adipose tissue (VAT) by GCs may be induced by several factors, including increased intake of high-caloric “comfort food” [7], increased lipoprotein lipase (LPL) activity particularly in VAT [8, 9], increased VAT adipogenesis through stimulation of differentiation of pre-adipocytes into mature adipocytes [10] and possibly by enhancing de novo lipogenesis [4, 11]. GC-induced increase in VAT is very relevant since VAT is well known to be associated with an unfavorable metabolic profile as opposed to subcutaneous adipose tissue (SAT) [12].

In contrast to their lipogenic actions, GCs are catabolic hormones and, as such, have lipolytic properties [4, 11]. In most performed in vitro studies, GCs increased lipolysis [4] by enhancing the activity of key lipolytic enzymes including adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) [13, 14]. It should be noted that differences were observed between adipose tissue depots with increased lipolysis in VAT as compared to SAT [15]. In addition, antilipolytic effects of GC treatment have also been observed in vitro [4]. In line with most of the in vitro observations, acute GC administration was shown to increase fasting whole-body lipolysis in healthy humans [16, 17], however, during more prolonged GC exposure, this effect was no longer present, most likely due to compensatory fasting hyperinsulinemia [18-20]. Indeed, we could recently show that a two-week treatment with prednisolone 7.5 mg did not increase, while prednisolone 30 mg daily even reduced basal lipolysis in healthy men [21]. However, in the same study, prednisolone treatment dose-dependently impaired insulin-stimulated suppression of lipolysis and thus increased plasma nonesterified fatty acids (NEFA) during hyperinsulinemia [21]. It is noteworthy that these effects occurred in the absence of significant changes in body weight, body composition, body fat distribution and liver fat content. Increased lipolysis and consequently enhanced plasma NEFA levels are detrimental since they impair glucose metabolism by reducing muscle and liver insulin sensitivity [22].
Finally, another mechanism by which GC-induced changes in adipose tissue may exert its diabetogenic effects is via alteration of the secretion of various adipokines, as was shown in *in vitro* studies [23]. An important limitation of the above-mentioned studies that have addressed the effects of GCs on adipose tissue function is that they were mostly carried out *in vitro* or *in vivo* in rodents, and no data are available from *in vivo* studies in humans. Therefore, in the present study, we assessed changes in SAT function and plasma levels of adipokines following a two-week treatment with low- or high dose prednisolone treatment in healthy men.

**RESEARCH DESIGN AND METHODS**

**Participants:** Twenty-four healthy normoglycemic Caucasian men (age 18-35 years, BMI 20-25 kg/m²) were recruited. Health status was confirmed by history, physical examination, screening blood tests and 75-g 2-h OGTT during the screening visit. Exclusion criteria were the presence of any disease, use of any medication, first-degree relative with type 2 diabetes, smoking, shift work, a history of glucocorticoid use, sport activities > twice/week and changes in weight in the 3 months prior to study participation. The study was approved by an independent ethics committee and the study was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent before participation.

**Study design:** Participants were randomly assigned to a 14-day treatment with prednisolone 7.5 mg once daily or prednisolone 30 mg once daily. At baseline and after two weeks of treatment, fasting blood samples were obtained, insulin sensitivity was measured by hyperinsulinemic-euglycemic clamp and subcutaneous adipose tissue biopsies were obtained both in the fasted state and following 45-min insulin infusion. Subjects were asked to refrain from drinking alcohol and to perform no strenuous exercise for a period of 48h before the study days.

**Hyperinsulinemic-euglycemic clamp:** A two-hour hyperinsulinemic-euglycemic clamp at 5.0 mM was performed to assess insulin sensitivity as described [24]. The mean glucose infusion rate during steady state (last 30 min of the clamp) was used to assess insulin sensitivity.

**Adipose tissue biopsy:** An abdominal subcutaneous adipose tissue biopsy was collected 6-8 cm lateral from the umbilicus under local anesthesia (2% lidocaine) by needle biopsy.
Adipose tissue was washed with sterile saline and was snap frozen in liquid nitrogen and stored at -80°C until analysis.

**Biochemical analyses:** Blood glucose concentrations were measured using an YSI 2300 STAT Plus analyzer (YSI, Yellow Springs, OH). Insulin levels were determined using an immunometric assay (Advia Centaur; Siemens Medical Solutions Diagnostics, Deerfield, IL). A single-plex human magnetic adiponectin and a three-plex human leptin, visfatin, and resistin assay (all from Biorad, Hercules, CA, USA) were used to determine serum levels of adiponectin, leptin, visfatin, and resistin using a Bioplex 200 suspension array system and Bioplex Pro II wash station (Biorad, Hercules, CA, USA) respectively. Adipokine concentrations were calculated from the appropriate optimized standard curves using Bio-Plex Manager software version 6.0 (Biorad). Intra- and interassay coefficients of variation for the Bioplex assays were both <4%. A commercial enzyme-linked immunosorbent assays (Elisa) was used to determine circulating RBP4 levels (R&D systems, Minneapolis, MN, USA). Intra- and interassay coefficients of variation for this assay were 8.1% and 8.6%, respectively.

**Western blot analysis:** Subcutaneous adipose tissue biopsies were homogenized in 50 mM Tris·HCl [pH 7.5], 150 mM NaCl, 0.5 % Triton X-100, 1 mM NaF, 1 mM Na3VO4, 2 mM MgCl2, 1 mM DTT, and protease inhibitors (Complete, Roche Diagnostics, Mannheim, Germany). Then, homogenates were cleared by centrifugation (15 min; 12,000 rpm; 4°C), and protein content was determined using Bradford reagent (Biorad Laboratories, München, Germany). Protein expression and phosphorylation was determined by Western blot analysis of ten microgram of protein using polyclonal antibodies recognizing ATGL, HSL, phospho-HSL-Ser660, protein kinase B (PKB)/Akt, phospho-Akt-Ser473, proline-rich Akt substrate (PRAS)40, phospho-PRAS40-Thr246, Krüppel-like factor (KLF)4, peroxisome proliferator activated receptor (PPAR)γ (all from Cell Signaling Technology, Danvers, MA, USA) or the insulin receptor β-subunit (Santa Cruz Biotechnology Santa Cruz, CA, USA). Bound antibodies were detected with HRP-conjugated secondary antibodies followed by enhanced chemiluminesence and visualized using a Versadoc system (Biorad). Blots were quantified using Quantity One software (version 4.6.9; Biorad).

**Statistical analyses:** Data are presented as mean ± standard deviation, or as median (interquartile range) in case of skewed distribution. Treatment effects were assessed by paired student t-test or Wilcoxon signed-rank test, comparing post treatment values and pre treatment values within each group. Univariate correlations were used to examine associations between parameters. Since these data concerned exploratory endpoints in our
study design, no formal power analysis was done. Calculations were done using SPSS 18.0 for Mac (Chicago, IL, USA). P<0.05 was considered to be statistically significant.

RESULTS

Participants: Baseline characteristics are presented in Table 1. All participants completed the study. One subject in the prednisolone 30 mg group complained of sleeplessness, which was mild and transient in nature. Otherwise, no side effects were reported in all treatment arms. Body weight was not altered by either prednisolone dose (Table 1).

Table 1. Baseline characteristics, and parameters of glucose metabolism and plasma adipokines before and during a two-week treatment with low- or high-dose prednisolone.

<table>
<thead>
<tr>
<th></th>
<th>Prednisolone 7.5 mg</th>
<th>Prednisolone 30 mg</th>
<th>P1</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
<td>Day 0</td>
<td>Day 14</td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>12</td>
<td>12</td>
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</tr>
<tr>
<td>Age, y</td>
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<td>-</td>
<td>21±2</td>
<td>-</td>
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<td>Weight, kg</td>
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<td>BMI, kg/m²</td>
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<td>21.4±2.2</td>
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<td>Waist, cm</td>
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<td>83±5</td>
<td>81±8</td>
<td>82±8</td>
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<tr>
<td>FPG, mmol/l</td>
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<td>4.7±0.3</td>
<td>4.5±0.2</td>
<td>4.9±0.4</td>
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<tr>
<td>OGTT glucose, mmol/l</td>
<td>3.9±0.9</td>
<td>-</td>
<td>3.6±0.8</td>
<td>-</td>
</tr>
<tr>
<td>FPI, pmol/l</td>
<td>36 (29-47)</td>
<td>36 (27-65)</td>
<td>32 (26-44)</td>
<td>56 (41-72)</td>
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<td>M-value, mg/kg.min</td>
<td>9.1 (7.4-10.6)</td>
<td>9.3 (4.6-11.1)</td>
<td>7.9 (5.0-9.6)</td>
<td>4.4 (2.6-6.4)</td>
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<td>Adiponectin, ng/ml</td>
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<td>11 (7-14)</td>
<td>20 (12-23)</td>
<td>12 (8-17)</td>
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<td>Resistin, ng/ml</td>
<td>4.8 (3.9-6.4)</td>
<td>5.1 (4.0-6.5)</td>
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<td>6.0 (4.4-7.6)</td>
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<td>Leptin, ng/ml</td>
<td>1.7 (0.6-3.6)</td>
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<td>RBP-4, mg/ml</td>
<td>42 (40-45)</td>
<td>37 (33-44)</td>
<td>41 (37-46)</td>
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<td>Visfatin, ng/ml</td>
<td>2.8 (2.2-3.3)</td>
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</table>

Data represent mean ± SD or median (interquartile range). Significance was tested by paired student t-test or wilcoxon signed-rank test. P1: Prednisolone 7.5 mg day 14 vs. day 0. P2: Prednisolone 30 mg day 14 vs. day 0. FPG: fasting plasma glucose; FPI: fasting plasma insulin; M-value: insulin sensitivity as measured by hyperinsulinemic-euglycemic clamp; OGTT: 2 hr glucose level 2 following ingestion of 75 g glucose.

Metabolic changes: Prednisolone 30 mg increased fasting plasma glucose and insulin levels and impaired clamp-measured insulin sensitivity, whereas prednisolone 7.5 mg only significantly increased fasting plasma glucose levels (Table 1).
Figure 1. Effects of glucocorticoids on adipose tissue function. Prednisolone 30 mg, but not prednisolone 7.5 mg treatment significantly impaired insulin signaling as measured by insulin-stimulated phosphorylation of Akt and proline-rich Akt substrate (PRAS)-40 (panel A+B). Both prednisolone dosages reduced the expression of adipogenesis markers peroxisome proliferator-activated receptor (PPAR)-γ and krüppel-like factor (KLF)-4 (panel C+D). Adipose triglyceride lipase (ATGL) expression was reduced by prednisolone 7.5 mg and 30 mg in the fasted state, but no differences were observed during insulin infusion (panel E). Neither fasting hormone sensitive lipase (HSL) expression (panel F) nor HSL phosphorylation by insulin was affected by prednisolone treatment (panel G). Adiponectin expression was reduced by both prednisolone dosages (panel H). Means±SD is shown. For panel A,B and G: white bar: pre treatment, fasting; black bar: pre treatment, during insulin infusion; bar with block-pattern: post treatment, fasting; grey bar: post treatment, during insulin infusion. For panel C, D, E, F and H: white bar: pretreatment, black bar: post treatment. *P<0.05;**P<0.01.

Adipokine plasma levels: Prednisolone 30 mg significantly increased levels of resistin (P=0.019) and leptin (P=0.002) from baseline, while the decrease in circulating levels of adiponectin was not statistically significant (P=0.07) (Table 1). Circulating levels of RBP-4 and visfatin were not significantly changed by prednisolone 30 mg treatment (P>0.05).
PRED7.5 mg did not alter the plasma levels of any of these adipokines (P>0.05) (Table 1). No associations were observed between prednisolone-induced changes in insulin sensitivity and changes in circulating levels of adipokines (data not shown).

**Adipose tissue insulin signaling:** Insulin infusion stimulated phosphorylation of PKB/Akt and its substrate PRAS40 as compared to the fasted state. This effect was reduced by prednisolone 30 mg treatment (Figure 1A+B). Although a trend was observed for the low dose group, this effect failed to reach statistical significance (Figure 1A+B).

**Adipose tissue markers of adipogenesis:** Both low-and high dose prednisolone treatment decreased the expression of markers of adipose tissue differentiation/adipogenesis PPAR-γ and KLF-4 (Figure 1C+D).

**Adipose tissue lipolytic enzymes:** Both prednisolone 7.5 mg and prednisolone 30 mg reduced the ATGL expression (Figure 1E), but did not alter HSL protein expression (Figure 1F). Insulin tended to lower HSL phosphorylation (P=0.06), which was not altered by low- or high-dose prednisolone treatment (Figure 1G).

**Adipose tissue adiponectin expression:** Both prednisolone 7.5 mg and prednisolone 30 mg reduced the expression of adiponectin (P<0.01 and P<0.05 respectively). Changes in adiponectin expression were correlated to changes in insulin signaling (pAkt phosphorylation; r=0.498; P=0.03) and adipose tissue differentiation (PPAR-γ expression; r=0.543; P=0.016).

**DISCUSSION**

This study is the first to show that prednisolone treatment impaired insulin signaling in SAT in vivo in humans. As such, the insulin-stimulated phosphorylation of well-known insulin target proteins including Akt/PKB and PRAS-40 was significantly impaired by high-dose prednisolone treatment, but was non-significantly reduced by low-dose prednisolone treatment.

These data confirm and expand studies done in vitro and in vivo in rodents, where GC treatment impaired insulin signaling [25] and where mice with reduced tissue GC levels (so called 11β-HSD-/- mice) were shown to have increased glucose uptake, insulin signaling and PPAR-γ expression during high-fat diet [26].
Impaired insulin action in adipose tissue may adversely affect both glucose and lipid metabolism. Indeed, an intact insulin signaling pathway is of pivotal importance for insulin-stimulated glucose uptake through recruitment of the glucose transporter (GLUT)-4 to the plasma membrane enabling glucose transport into the cell [27]. Although the overall contribution of glucose uptake by adipose tissue in the postprandial state is thought to be relatively modest [28], it does contribute to glucose tolerance as was elegantly demonstrated in adipose tissue-specific glut4−/− mice [29]. These mice developed hyperglycemia and were characterized by liver and skeletal muscle insulin resistance, most likely due to altered secretion of adipokines [29]. In our study, prednisolone 30 mg altered the secretion of various adipokines towards a more diabetogenic profile. Thus, plasma concentrations of adiponectin, which are generally positively associated with insulin sensitivity, showed a tendency toward reduced levels following high-dose prednisolone treatment, whereas adiponectin protein expression in SAT was decreased by both prednisolone dosages. The adipokines resistin and leptin, which are usually increased in insulin resistant states were both increased by prednisolone 30 mg. Although increased leptin levels has been shown to inhibit food intake in the CNS, leptin also acts as pro-inflammatory factor in adipose tissue by promoting the release of pro-inflammatory adipokines. Similarly, resistin has also been demonstrated to promote circulating pro-inflammatory factors derived from adipose tissue [30].

In addition to promoting glucose uptake in adipose tissue, insulin is a key hormone in the regulation of lipid metabolism, amongst others by inhibiting adipose tissue lipolysis and thus decreasing plasma NEFA levels [31]. In contrast to the effects of insulin, GCs increase fasting lipolysis rates when administered acutely [16, 17]. During more prolonged GC exposure, however, this lipolytic effect is no longer observed most likely due to compensatory hyperinsulinemia [18-21]. In our recent study, high-dose prednisolone treatment even lowered fasting lipolysis [21]. In line with these findings, we observed decreased fasting ATGL expression, the rate-limiting enzyme in adipose tissue lipolysis, during both low- and high-dose prednisolone treatments, while fasting HSL expression was unchanged.

As stated previously, GCs impaired the suppressive effects of insulin on whole-body lipolysis [21]. Since both ATGL expression and phosphorylation of HSL during insulin infusion were unaltered by prednisolone treatment, it is unclear through which mechanisms this effect occurs. Possibly, the inhibiting effects of insulin on protein kinase A and perilipin expression, a lipid droplet-associating protein, are reduced by prednisolone treatment, thereby promoting triglyceride lipolysis [13]. An alternative hypothesis is that the increment in whole-body lipolysis is mostly derived from VAT, from which no biopsy could be obtained in the present...
study design. Importantly, increased lipolysis levels impair glucose homeostasis by reducing muscle and liver insulin sensitivity [22].

In addition to changing adipose tissue function, GCs are known to alter adipose tissue distribution during more prolonged treatment by augmenting central fat deposition with wasting of peripheral fat depots. Although two weeks is a relatively short treatment period, and no significant alterations were shown in body fat distribution in a previous study [21], we observed decreased markers of adipogenesis in SAT, compatible with the observed phenotype after more prolonged GC exposure. The GC-induced reduction in SAT with concomitant increase in VAT is clinically relevant since VAT is well known to be associated with an unfavorable metabolic profile as opposed to SAT and is associated with increased cardiovascular risk [12]. Importantly, a number of effects were already observed during treatment with prednisolone 7.5 mg daily, a low dose commonly used in clinical practice for prolonged periods. Many patients treated with GCs in clinical practice suffer from systemic inflammatory conditions, which may also be associated with impaired adipose tissue function [32]. Therefore, the effects of GC treatment on adipose tissue function may be more complex since GCs reduce systemic inflammation and require further investigation.

In conclusion, this study demonstrates for the first time that prednisolone treatment impaired SAT function by impairing insulin signaling and reducing adipogenesis in vivo in healthy humans. In addition, plasma levels of adiponectin, leptin and resistin were changed to a more diabetogenic profile. Further studies in humans are warranted to study the mechanisms that may underly the effects of GCs on adipose tissue function.

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REFERENCES


