Chapter 10

Glucocorticoid Treatment Impairs Microvascular Function in Healthy Males, in Association with its Adverse Effects on Glucose Metabolism and Blood Pressure

ABSTRACT

Glucocorticoids (GCs) are anti-inflammatory agents but also induce side effects, including insulin resistance, glucose intolerance and hypertension. In this study, we investigated the role of microvascular function in the development of these adverse effects. In a randomized, placebo-controlled, double-blind, dose-response intervention study, 32 healthy males (age: 21±2 years; BMI: 21.9±1.7 kg/m²) were allocated to prednisolone 30 mg once daily (n=12), prednisolone 7.5 mg once daily (n=12), or placebo (n=8) for two weeks. Compared to placebo, prednisolone treatment dose-dependently decreased capillary recruitment. Additionally, prednisolone treatment dose-dependently impaired insulin sensitivity and glucose tolerance, but only prednisolone 30 mg increased systolic blood pressure. Prednisolone-induced changes in insulin-stimulated capillary recruitment were associated with insulin sensitivity (r=+0.76), postprandial glucose levels (r=−0.52) and systolic blood pressure (r=−0.62). To further investigate underlying mechanisms, male Wistar rats were treated with prednisolone or saline for 7 days. Prednisolone treatment attenuated the vasodilatory actions of insulin in muscle resistance arteries by impairing insulin signaling through the Akt pathway. We conclude that prednisolone dose-dependently impaired insulin-stimulated capillary recruitment by inducing vascular insulin resistance through direct interference with vascular insulin signaling. We propose that GC-induced impairments of microvascular function may contribute to the adverse effects of GC treatment on glucose metabolism.

Clinical Trial Registration Number: ISRCTN 78149983
Glucocorticoids (GCs) represent the most important and frequently used class of anti-inflammatory drugs, but their use is hampered by an increased risk of serious side effects, particularly hypertension, insulin resistance, reduced glucose tolerance and overt diabetes in susceptible individuals [1-5]. Although the exact mechanisms underlying these adverse effects remain to be identified, GC-induced insulin resistance may underlie part of these side effects [6]. Insulin resistance is characterized by the diminished ability of insulin to initiate intracellular signaling, primarily in liver, adipose tissue and skeletal muscle [7]. Impaired insulin signaling in these tissues results in reduced glucose uptake, insufficient suppression of hepatic glucose output, compensatory hyperinsulinemia and dyslipidemia, which collectively constitute the so-called metabolic syndrome [7].

Insulin action is not restricted to metabolically active tissues. Recently, it has become clear that vascular tissue, and particularly endothelial cells, represents an important physiological target for insulin and a significant regulator of overall insulin-stimulated glucose uptake [8-10]. Insulin promotes its own access to muscle interstitial space by increasing blood flow and by recruiting capillaries to expand the endothelial transporting surface available for nutrient exchange. In addition, the local actions of insulin on (micro)vascular tissue may be of relevance to the pathogenesis of hypertension, an important feature of the metabolic syndrome [8, 10-12]. Insulin exerts a vasodilatory action by promoting nitric oxide (NO) release from the endothelial cells. This involves the phosphorylation of endothelial NO synthase (eNOS), which on its turn is mediated by the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [13]. Concomitantly, insulin activates extracellular signal-regulated kinase (ERK)1/2 signaling in endothelial cells, which results in enhanced expression of the vasoconstrctor regulator endothelin-1 [10, 13]. In healthy subjects, the vasodilatory signal prevails, but if activation of the PI3K/Akt/eNOS pathway is impaired, as observed in insulin resistant states, insulin-regulated vasodilatation becomes compromised. In this manner, vascular insulin resistance may contribute to the development of hypertension and impaired whole-body insulin-stimulated glucose uptake [14].

It is presently unclear whether vascular insulin resistance could be involved in the unfavorable effects of GCs on glucose metabolism and blood pressure. Thus, in the present study, we investigated whether the metabolic effects of the GC prednisolone, given at both a clinically relevant low- and high dosage for 2 weeks, would concomitantly induce vascular insulin resistance in healthy humans. Subsequently, we assessed associations among prednisolone-induced vascular insulin resistance and changes in blood pressure, whole-body insulin-mediated glucose uptake and glucose tolerance. In order to detail underlying mechanisms, vasoactivity and Akt-signaling and MAPK pathway signaling in the endothelium were examined in cremaster arterioles of Wistar rats treated with prednisolone.
METHoDS

Clinical study
Participants: Thirty-two healthy Caucasian subjects were recruited by local advertisement. Inclusion criteria were: age between 18-35 years, body mass index (BMI) between 20.0-25.0 kg/m^2, good physical health (determined by medical history, physical examination and screening blood tests) and normoglycemia as defined by fasting plasma glucose (FPG) < 5.6 mmol/L and 2-hour glucose < 7.8 mmol/L following a 75 g oral glucose tolerance test (OGTT), performed at screening visit. Exclusion criteria were any previous or current illness, use of any medication, first-degree relative with type 2 diabetes, smoking, shift work, a history of GC use, excessive sports activities (i.e. more often than two times/week) and recent changes in weight or physical activity. 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: The study was a randomized, placebo-controlled, double blind, dose-response intervention study. Following assessment of eligibility and baseline measurements, participants were randomized to receive either prednisolone 30 mg once daily (n=12), prednisolone 7.5 mg once daily (n=12), or placebo treatment (n=8) for a period of 14 days using block randomization, as carried out by the Department of Experimental Pharmacology of the VU University Medical Center. Prior to treatment and on day 13 of treatment, microvascular function was measured by capillary microscopy in the fasted state and during insulin infusion. Insulin sensitivity was determined by hyperinsulinemic-euglycemic clamp. Prior to treatment and on day 14 of treatment, a standardized consecutive meal challenge test was performed to assess glucose tolerance. All measurements were conducted in a temperature-controlled room (23.4±0.4°C) starting at 0730 AM, following a 12-h overnight fast with the subjects in the supine position. Subjects refrained from drinking alcohol for a period of 24h before the study day and did not perform strenuous exercise for a period of 48h before each study day. Baseline measurements were obtained following 30 min of rest and acclimatization. During all visits, including a follow-up visit at day 7 of treatment, safety and tolerability were assessed (Supplementary Figure 1). A patient flow diagram is shown in Supplementary Figure 2.

Capillary microscopy: Nail fold capillary studies were performed in the fasted state (T=-120 min) and during hyperinsulinemia (T=120 min) as described previously [15] by a single experienced investigator (Figure 1). Baseline capillary density was defined as the number of continuously erythrocyte-perfused capillaries per square millimeter of nail fold skin. Postocclusive reactive hyperemia after 4 min of arterial occlusion was used to assess functional
capillary recruitment. Capillary recruitment was calculated by dividing the increase in perfused capillary density during postocclusive reactive hyperemia by the baseline perfused capillary density. In addition, venous congestion to expose a maximal number of capillaries was done. All procedures were performed twice, and the mean of both measurements was used for analyses. The day-to-day coefficient of variation of functional capillary recruitment was 15.9±8.0 % as determined in 10 healthy individuals on 2 separate days. Skin temperature was monitored during the tests. One subject in the prednisolone 7.5 arm could not be analyzed due to insufficient quality of microscopic images during treatment with study medication and was left out of the analyses.

Blood pressure: Systolic and diastolic blood pressure were manually measured (Welch Allyn, Delft, Netherlands) by a single experienced investigator following the 30 min acclimatization period. The average of three consecutive blood pressure measurements with 5-min interval was used for further analyses.

Figure 1. Design of the experimental protocol. A two-hour hyperinsulinemic-euglycemic clamp was performed for 180 min. Microcirculation: nailfold capillary microscopy; BP: blood pressure measurements; biopsy: percutaneous skeletal muscle biopsy. Arrows indicate measurement of plasma insulin levels.

Hyperinsulinemic-euglycemic clamp: Whole-body insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamp method as described previously [16]. Briefly, a primed, continuous (40 mU/min) insulin infusion (Actrapid 100 IU/mL; Novo Nordisk, Bagsvaerd, Denmark) was given for 120 min; plasma glucose was kept at 5 mmol/l by adjusting the rate of a 20% glucose infusion based on plasma glucose measurements performed at 5-min intervals (Figure 1). Whole-body insulin sensitivity was quantified by the M-value (mg/kg/min), calculated between min 90-120 during steady-state insulin concentrations as described previously [16].
**Standardized meal test:** Glucose tolerance was assessed following two consecutive identical meals that were served as breakfast at 09.00 AM and as lunch at 1.00 PM. The meals contained 905 kcal (50 g fat, 75 g carbohydrates, 35 g protein) each. Samples for determination of glucose were obtained at 30 min intervals, with the meal beginning immediately after the time 0 sample and consumed within 15 min. Area under the postprandial glucose curve (AUC) was calculated by the trapezoid method. Insulin sensitivity in the postprandial state was calculated as the oral glucose sensitivity index (OGIS) [17].

**Insulin signaling in skeletal muscle biopsies:** Since skeletal muscle tissue represents the major site of glucose clearance in the postprandial state, characterized by a classic intracellular insulin signaling cascade, we assessed the effects of prednisolone on key proteins in this cascade in skeletal muscle tissue. To this end, a percutaneous skeletal muscle biopsy was taken from the vastus lateralis muscle using a Bergstrom needle [18] under local anesthesia with lidocaine 1% (B. Braun, Melsungen, Germany) before and after 30 min of insulin infusion. Muscle biopsies were washed with sterile NaCl 0.9% to reduce blood contamination and were thereafter snap-frozen in liquid nitrogen and stored at -80°C for further analysis. Western blotting of proteins involved in insulin signaling, including phosphorylation of Akt and its downstream substrate proline-rich Akt substrate of 40 kDa (PRAS40) was done as described previously [19]. Antibodies for PRAS40, Phospho-Akt-Ser473 and Tubulin were obtained from Cell Signaling Technology (Boston, MA), for total Akt from Millipore Corporation (Billerica, MA), for the insulin receptor from Santa Cruz Biotechnology Inc (Santa Cruz, CA) and for Phospho-PRAS40-Thr246 from Biosource (Carlsbad, CA).

**Study medication:** Prednisolone tablets were purchased from Pfizer AB (Sollentuna, Sweden) and placebo tablets were obtained from Xendo Drug Development (Groningen, The Netherlands). Tablets were capsulated in order to allow the treatment to be blinded [20]. Study medication was taken at 08.00 AM during the two-week treatment except for day 13 and day 14, when it was ingested at 06.00 AM. Patients kept a diary in which the exact time of medication intake during the study was registered.

**Biochemical methods:** 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, Gentautz Siemens Medical Solutions Diagnostics, USA).

**Statistical analyses:** Data are presented as mean values ± standard deviation (SD), or as median (interquartile range) in case of skewed distribution. Absolute changes from baseline were calculated (on treatment value minus pre treatment value) for all parameters. Non-parametric analysis was chosen due to uneven sample size in the different groups and the
relatively small numbers in each group. Baseline values were compared between the groups by Kruskal-Wallis. For treatment-induced effects, Kruskal-Wallis with trend analysis was performed (the Jonckheere-Terpstra test). Only in case of a significant finding, prednisolone 7.5 mg and prednisolone 30 mg were compared against placebo by posthoc testing, using the Mann-Whitney U test. To correct for multiple testing, Bonferroni correction was applied. Correlations between the various parameters were assessed with Spearman correlations. All statistical analyses were run using SPSS version 18.0 for Mac OS X (Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

**Animal Study**

**Animals and treatments:** The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. The local ethics committee for animal experiments approved the procedures. Male Wistar rats (300-350 g; Charles-River, Maastricht, the Netherlands) were treated with prednisolone (8 mg/kg per day; $n=6$) or saline ($n=6$) by intraperitoneal injection for 7 days. Two hours after the final injection, the rats were sacrificed by isoflurane overdose and resistance arteries of the cremaster muscles were isolated as described previously [21].

**Vasoreactivity experiments:** After dissection, a first order cremaster arteriole was placed in a pressure myograph. Only vessels that developed substantial spontaneous constriction (>40% of the passive diameter) to pressure (65 mmHg) and then showed a clear vasodilator response (>10% of the diameter) to the endothelium-dependent vasodilator acetylcholine (0.1 mm; Sigma) were used [21]. Acute effects of insulin (Novo Nordisk, Alphen a/d Rijn, the Netherlands) on the diameter were studied at three different concentrations: 0.02, 0.2 and 2 nmol/l, and diameter changes at each concentration were recorded for 30 min.

**Western blots:** Segments of cremaster arteries from the same rat were exposed to insulin or solvent for 15 min at 34°C as described [22]. The protein lysates were stained with a specific antibody against phosphorylated Akt, total Akt, phosphorylated ERK1/2 and total ERK1/2 (Cell Signaling Technology, Boston, MA) and visualized with a chemiluminescence kit (Amersham). Differences in phosphorylated protein were adjusted for differences in the corresponding total protein staining or actin.

**Statistical analyses:** Steady-state responses are reported as mean changes in diameter from baseline (in percent) ± SEM. The baseline diameter was defined as the arterial diameter just before addition of the first concentration of insulin. Western blot data were expressed as relative to unstimulated controls, assigning a value of 1 to the control. Differences between
the various parameters were assessed by the Mann-Whitney U test. A \( P < 0.05 \) was considered statistically significant.

RESULTS

Clinical study

Baseline characteristics and anthropometrics: Prior to treatment, all treatment groups had comparable anthropometrics, blood pressure and glucose tolerance (Table 1). Treatment with study medication did not alter body weight (Table 2).

Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Prednisolone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.5 mg</td>
<td>30 mg</td>
</tr>
<tr>
<td>N</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Age (y)</td>
<td>21±3</td>
<td>21±2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>75±8</td>
<td>75±6</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>186±7</td>
<td>184±6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.6±1.5</td>
<td>22.1±1.5</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>126±7</td>
<td>124±9</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>79±8</td>
<td>79±6</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>4.4±0.3</td>
<td>4.4±0.2</td>
</tr>
<tr>
<td>2-hr glucose OGTT (mmol/l)</td>
<td>3.7±1.0</td>
<td>3.9±0.9</td>
</tr>
</tbody>
</table>

Means±SD are provided. Significance was tested by Kruskal-Wallis. No statistically significant differences were observed between the groups at baseline. 2-hr glucose OGTT denotes plasma glucose concentrations 2 h after ingestion of 75 g glucose during an oral glucose tolerance test.

Prednisolone impaired capillary recruitment: In the fasted state prior to insulin infusion, prednisolone treatment did not affect baseline capillary density, capillary recruitment or maximal number of capillaries following venous congestion (Figure 2A; Table 3). Insulin infusion increased capillary recruitment by 13±4% (\( P < 0.001 \)), but did not affect baseline capillary density (Table 3). During insulin infusion, prednisolone treatment did not affect baseline capillary density, however, prednisolone reduced functional capillary recruitment by 9±4% (prednisolone 7.5 mg; \( P = 0.18 \)) and 17±3% (prednisolone 30 mg; \( P = 0.002 \)) as compared to placebo (Figure 2B, Table 3).

Prednisolone increased systolic blood pressure: Systolic blood pressure, but not diastolic blood pressure, was raised by prednisolone 30 mg (6±1.2 mmHg, versus placebo \( P = 0.006 \)). Prednisolone 7.5 mg did not affect blood pressure (Table 2).
Table 2. Metabolic parameters and blood pressure before and during treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-treatment</th>
<th>On-treatment</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
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<tr>
<td>Weight (kg)</td>
<td>PLB 75±8</td>
<td>76±7</td>
<td>0.539</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>7.5 75±6</td>
<td>75±6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>30  73±10</td>
<td>74±10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>PLB 126±7</td>
<td>123±9</td>
<td>0.002</td>
<td>0.710</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>7.5 124±9</td>
<td>124±11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30  120±9</td>
<td>124±8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>PLB 79±8</td>
<td>76±7</td>
<td>0.042</td>
<td>1.0</td>
<td>0.164</td>
</tr>
<tr>
<td></td>
<td>7.5 79±6</td>
<td>76±6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>30  78±10</td>
<td>80±9</td>
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<td></td>
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<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>PLB 4.4±0.3</td>
<td>4.5±0.3</td>
<td>0.04</td>
<td>0.418</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>7.5 4.4±0.2</td>
<td>4.7±0.3</td>
<td></td>
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<tr>
<td></td>
<td>30  4.5±0.2</td>
<td>4.9±0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting plasma insulin (pmol/l)</td>
<td>PLB 31 (28-40)</td>
<td>33 (28-39)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>7.5 36 (29-47)</td>
<td>36 (27-65)</td>
<td>0.001</td>
<td>1.0</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>30  32 (26-44)</td>
<td>56 (41-72)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area under glucose curve (mmol/l.hour)</td>
<td>PLB 2197±193</td>
<td>2182±92</td>
<td>&lt;0.001</td>
<td>0.008</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>7.5 2250±121</td>
<td>2492±127</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>30  2189±228</td>
<td>2734±260</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>OGIS (ml/min/m²)</td>
<td>PLB 488±50</td>
<td>489±33</td>
<td>&lt;0.001</td>
<td>0.01</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>7.5 477±41</td>
<td>420±42</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>30  484±52</td>
<td>375±75</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>M-value (mg/kg.min)</td>
<td>PLB 7.0 (4.7-11.1)</td>
<td>9.0 (6.5-12.8)</td>
<td>&lt;0.001</td>
<td>0.062</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>7.5 9.1 (7.4-10.6)</td>
<td>9.3 (4.6-11.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30  7.9 (5.8-9.6)</td>
<td>4.4 (2.6-6.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp glucose levels (mmol/l)</td>
<td>PLB 5.0±0.3</td>
<td>5.0±0.2</td>
<td>0.978</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>7.5 5.0±0.1</td>
<td>5.0±0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30  5.0±0.1</td>
<td>5.1±0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp insulin levels (pmol/l)</td>
<td>PLB 441±81</td>
<td>412±49</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>7.5 488±61</td>
<td>453±69</td>
<td>0.140</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td></td>
<td>30  504±78</td>
<td>460±81</td>
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</table>

Data are mean±SD or median (interquartile range). Between-group changes from baseline were tested by Kruskal-Wallis with trend analysis (P1). In case of a significant finding, post hoc testing (by Mann-Whitney U) was done (P2 to compare prednisolone 7.5 vs. placebo and P3 to compare prednisolone 30 mg vs. placebo, respectively). Treatment groups: 7.5=prednisolone 7.5 mg daily; 30=prednisolone 30 mg daily; PLB=placebo. Abbrevations: M-value: insulin sensitivity as measured by the hyperinsulinemic-euglycemic clamp; NA: not applicable; OGIS: postprandial insulin sensitivity.
Figure 2. The effects of prednisolone (PRED) on capillary recruitment. Capillary recruitment in the fasted state was not impaired by either prednisolone dosage (A), however, prednisolone treatment dose-dependently impaired insulin-stimulated microvascular function (B). Data represent absolute change from baseline; mean ± SEM are provided. White bars: placebo; grey bars: prednisolone 7.5 mg daily; black bars: prednisolone 30 mg daily. Between-group changes from baseline were tested by Kruskal-Wallis with trend analysis (indicated by top line). Posthoc tests were done by Mann-Whitney U with Bonferroni correction for multiple testing (indicated by line with brackets). ***p<0.001,**p<0.01.

Figure 3. The effect of prednisolone (PRED) treatment on phosphorylation of proteins involved in insulin signaling in skeletal muscle. Prednisolone treatment did not significantly alter phosphorylation of Akt (A) and proline-rich Akt substrate of 40 kDa (PRAS40) (B) in the basal state or following 30 min of insulin infusion when compared between the 3 treatment groups. Prior to treatment, insulin increased phosphorylation of PRAS40. After treatment, however, this effect was no longer significant in the prednisolone 30 mg group, indicating reduced effects of insulin stimulation (B). A representative blot is shown in (C). Data indicate ratio between phosphorylated and total protein content in arbitrary units; mean ± SEM are provided. White bars: fasting state; black bars: insulin infusion. Pre indicates baseline measurements for all participants; PLB: placebo; 7.5: prednisolone 7.5 mg once daily; 30: prednisolone 30 mg once daily. **p<0.01,*p<0.05,#p=0.2.
Table 3. Capillary density before and during treatment.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>On-treatment</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline density fasting</td>
<td>PLB</td>
<td>37.6±3.9</td>
<td>40.9±5.1</td>
<td>0.688</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>39.4±2.9</td>
<td>40.2±4.3</td>
<td>0.688</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>40.1±6.3</td>
<td>41.8±6.6</td>
<td>0.688</td>
<td>NA</td>
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<tr>
<td>Peak density fasting</td>
<td>PLB</td>
<td>47.6±5.4</td>
<td>52.1±6.0</td>
<td>0.189</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>51.3±5.6</td>
<td>54.3±7.1</td>
<td>0.189</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>52.0±8.9</td>
<td>54.8±9.8</td>
<td>0.189</td>
<td>NA</td>
</tr>
<tr>
<td>Capillary recruitment</td>
<td>PLB</td>
<td>26.6±2.8</td>
<td>26.4±8.4</td>
<td>1.0</td>
<td>NA</td>
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<tr>
<td>fasting (%)</td>
<td>7.5</td>
<td>30.0±5.8</td>
<td>30.8±7.7</td>
<td>1.0</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>29.7±6.6</td>
<td>28.5±8.5</td>
<td>1.0</td>
<td>NA</td>
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<tr>
<td>Venous occlusion</td>
<td>PLB</td>
<td>51.1±6.1</td>
<td>54.5±6.4</td>
<td>0.742</td>
<td>NA</td>
</tr>
<tr>
<td>(n/mm²)</td>
<td>7.5</td>
<td>59.6±7.8</td>
<td>60.0±7.6</td>
<td>0.742</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>57.0±7.9</td>
<td>60.3±10.6</td>
<td>0.742</td>
<td>NA</td>
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<tr>
<td>Baseline density</td>
<td>PLB</td>
<td>37.1±2.1</td>
<td>41.4±6.5</td>
<td>0.001</td>
<td>0.03</td>
</tr>
<tr>
<td>hyperinsulinemia (n/mm²)</td>
<td>7.5</td>
<td>39.3±4.0</td>
<td>41.0±5.6</td>
<td>0.001</td>
<td>0.03</td>
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<td>30</td>
<td>39.1±6.5</td>
<td>41.7±7.4</td>
<td>0.001</td>
<td>0.03</td>
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<tr>
<td>Peak density</td>
<td>PLB</td>
<td>51.3±2.2</td>
<td>60.8±8.1</td>
<td>0.011</td>
<td>0.03</td>
</tr>
<tr>
<td>hyperinsulinemia (n/mm²)</td>
<td>7.5</td>
<td>57.1±8.0</td>
<td>57.9±8.4</td>
<td>0.011</td>
<td>0.03</td>
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<tr>
<td></td>
<td>30</td>
<td>55.1±9.7</td>
<td>54.4±10.3</td>
<td>0.011</td>
<td>0.03</td>
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<tr>
<td>Capillary recruitment</td>
<td>PLB</td>
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<td>44.8±9.8</td>
<td>0.001</td>
<td>0.18</td>
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<tr>
<td>hyperinsulinemia (%)</td>
<td>7.5</td>
<td>44.9±8.2</td>
<td>41.5±12.9</td>
<td>0.001</td>
<td>0.18</td>
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<tr>
<td></td>
<td>30</td>
<td>41.2±5.2</td>
<td>30.6±8.2</td>
<td>0.001</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Data are mean±SD or median (interquartile range). Between-group changes from baseline were tested by Kruskal-Wallis with trend analysis (P1). In case of a significant finding, posthoc testing (by Mann-Whitney U) was done (P2 to compare prednisolone 7.5 mg vs. placebo and P3 to compare prednisolone 30 mg vs. placebo, respectively). Treatment groups: 7.5=prednisolone 7.5 mg daily; 30=prednisolone 30 mg daily. Abbrevations: NA: not applicable.

Prednisolone dose-dependently impaired glucose metabolism and insulin sensitivity:
Prednisolone 30 mg, but not prednisolone 7.5 mg increased FPG levels relative to placebo (P=0.04), despite fasting hyperinsulinemia (P=0.008) (Table 2). As compared to placebo, glucose tolerance during the standardized meal test was dose-dependently reduced by prednisolone treatment: glucose levels, measured as area under the curve (AUC), increased by 11±5% and 27±9%, for prednisolone 7.5 mg and Prednisolone 30 mg respectively (Table 2). Clamp-measured whole-body insulin sensitivity was decreased by prednisolone treatment as compared to placebo in a dose-dependent manner: mean differences: -2.1±0.8 mg/kg.min for prednisolone 7.5 mg and -4.5±0.7 mg/kg.min for prednisolone 30 mg. Similarly, postprandial insulin sensitivity calculated by OGIS, was dose-dependently reduced by prednisolone treatment as compared to placebo (Table 2).
Figure 4. Associations among prednisolone (PRED)-induced changes in insulin-stimulated microvascular function, and PRED-induced changes in metabolic and vascular parameters, including fasting plasma glucose (A), area under the postprandial glucose curve (B), insulin-stimulated glucose disposal (C), postprandial insulin sensitivity (D) and systolic blood pressure (E). Correlations were assessed with Spearman correlations.
Prednisolone and insulin signaling in skeletal muscle biopsies: As compared to placebo, prednisolone treatment did not result in significant reduction in phosphorylation of Akt (Figure 3A) and PRAS40 (Figure 3B) before and during insulin infusion. Of note, when within-group changes were assessed, the characteristic insulin-mediated increase in PRAS-phosphorylation as observed prior to treatment, was no longer present following high-dose prednisolone treatment (Figure 4B), indicating reduced insulin-stimulated phosphorylation.

Associations among insulin-stimulated capillary, metabolic parameters and blood pressure: Prednisolone-induced reductions in insulin-stimulated capillary recruitment were negatively associated with changes in fasting (P<0.0001) (Figure 4A) and postprandial glucose levels (P=0.003) (Figure 4B), while positive associations were found with impaired clamp-measured insulin sensitivity (P<0.0001) (Figure 4C) and postprandial insulin sensitivity (P=0.004) (Figure 4D). In multiple regression analyses, adjusting for age and BMI, changes in insulin-stimulated capillary recruitment statistically explained approximately 38% of the prednisolone-induced changes in insulin-mediated glucose disposal (β was reduced from -0.695 to -0.434). Finally, prednisolone-induced changes in insulin-stimulated capillary recruitment were negatively correlated with the observed rise in systolic blood pressure (Figure 4E).

Safety and tolerability: One subject in the prednisolone 30 mg group complained of sleeplessness, which was mild and transient of nature. Otherwise, no side effects were reported in all treatment arms.

Animal study
Prednisolone impaired insulin-stimulated vasodilation: To assess whether prednisolone directly impaired microvascular function in muscle and to further elucidate the mechanisms involved, healthy Wistar rats were treated with prednisolone. General characteristics of resistance arteries were similar in the prednisolone and saline-treated rats. Passive intraluminal diameter, basal tone and endothelium-dependent vasodilation of the arterioles following acetylcholine stimulation were not affected by prednisolone treatment (Supplemental Table 2 and Supplemental Figure 3). Insulin dose-dependently induced vasodilation, which was impaired by prednisolone treatment at all measured concentrations (Figure 5A).

Prednisolone reduced vascular akt signaling: Basal phosphorylation of Akt in cremaster resistance arteries was not changed by prednisolone treatment. However, prednisolone
reduced the insulin-stimulated increase in Akt phosphorylation (Figure 5B). Basal and insulin-stimulated phosphorylation of ERK1/2 were not affected by prednisolone treatment (Figure 5C).

**Figure 5. The effects of prednisolone treatment on isolated rat cremaster resistance arterioles.** Prednisolone treatment impaired the vasoactive actions of insulin *ex vivo* (A). Prednisolone impaired insulin-stimulated phosphorylation of Akt (B), but did not affect phosphorylation of ERK1/2 (C). A representative blot is shown in (D). Data indicate percentual change from baseline for vasoreactivity experiments (A) and the ratio between phosphorylated and total protein content in arbitrary units for Western blotting experiments (B) and (C); mean ± SEM are provided. White bars: fasting state; black bars: insulin infusion. Between-group changes from baseline were tested by Mann-Whitney-U test. **p<0.01, *p<0.05.**
DISCUSSION

This is the first study describing a dose-dependent impairment of insulin-stimulated microvascular function following GC treatment in healthy individuals, as a possible mechanism contributing to the concomitantly occurring decrease in insulin sensitivity and glucose tolerance and elevation of blood pressure. These findings were substantiated by the demonstration of reductions in insulin-stimulated vasodilation through impaired insulin-mediated phosphorylation of Akt, but unchanged MAPK pathway signaling in rat resistance arteries. These findings are consistent with a role for vascular insulin resistance in the development of GC-related adverse metabolic effects.

As observed previously [6, 20], a two-week treatment with prednisolone dose-dependently reduced whole-body insulin sensitivity and glucose tolerance, while high-dose prednisolone treatment significantly increased systolic blood pressure. An additional confirmatory finding of the present study is the identification of the vasoactive properties of insulin, as insulin increased capillary recruitment by 13% in these healthy subjects. A novel finding was that prednisolone dose-dependently impaired these vasodilatory actions of insulin in vivo, while prednisolone treatment did not affect microvascular function in the fasted state. It has been reported previously that GCs particularly impair metabolism under stimulated conditions, i.e. during hyperinsulinemic-euglycemic clamps or in the postprandial state. Accordingly, we have recently shown that prednisolone dose-dependently impaired metabolic fluxes during insulin infusion, but not in the fasted state when prednisolone induced fasting hyperinsulinemia [6]. Moreover, fasting glucose levels are often only mildly elevated by GC treatment, whereas during the day significant hyperglycemia develops, especially in the postprandial period [20, 23]. This seems also the case for the vascular effects of prednisolone, because in the present study we did not observe prednisolone-induced changes in capillary recruitment in the fasted state, when prednisolone augmented fasting insulin levels. Collectively, the data demonstrated that prednisolone induced vascular insulin resistance.

Subsequently, we analyzed whether vascular insulin resistance was related to the prednisolone-induced impairment of glucose metabolism and blood pressure rise. Indeed, prednisolone-induced impairment in capillary recruitment was significantly associated with insulin-stimulated glucose disposal during the hyperinsulinemic-euglycemic clamp, as well as to postprandial glucose levels and postprandial insulin sensitivity, suggesting that capillary recruitment may also be an important factor contributing to postprandial glucose metabolism. In line with this finding, Keske and colleagues recently demonstrated using contrast-enhanced ultrasound that in obese, insulin resistant subjects, capillary recruitment in the postprandial state was impaired [24].

[24]
Additionally, the approximately 6 mmHg increase in systolic blood pressure induced by high-dose prednisolone treatment was related to the decrease in insulin-stimulated capillary recruitment. Previously, various mechanisms have been proposed to underlie the GC-induced increase in blood pressure, including salt and water retention, altered sympathovagal balance, increased responsiveness to pressor stimuli such as catecholamines and angiotensin II and reduced NO availability [25]. We suggest that GC-induced vascular insulin resistance with impaired insulin signaling via PI3K/Akt to eNOS may be an alternative mechanism to explain the increase in blood pressure observed during GC treatment. A similar concept has been proposed for the development of hypertension in obesity and other insulin resistant states [10-12]. However, the contribution of insulin signaling to eNOS in the regulation of blood pressure in different states of insulin resistance is not unequivocal [12].

Although it seems very likely that prednisolone treatment also directly affects insulin signaling in skeletal muscle tissue, we did not detect altered insulin signaling as measured by phosphorylated Akt and its substrate PRAS40 in skeletal muscle biopsies obtained in the healthy participants, when compared to placebo. In rat skeletal muscle, it has been shown previously that the GC dexamethasone impaired various components of the insulin signaling cascade, including decreased phosphorylation of IRS-1, PI-3K and Akt, resulting in impaired translocation of glucose transporter (GLUT) 4 to the plasma membrane [26-30]. These seemingly discrepant findings may not be easily explained. Since we did find statistically significant changes when within-group comparisons were made, we may have been limited by the relatively small size of the study. Furthermore, since proteins remain in a phosphorylated state for a limited time period, the timing of biopsy collection, i.e. at 30 min following onset of hyperinsulinemia, may have influenced our findings. Taken together, our data are compatible with an important role for prednisolone-induced vascular insulin resistance in the reduction in glucose uptake by skeletal muscle during hyperinsulinemia and in the postprandial state following prednisolone treatment.

Since muscle tissue is the main peripheral site of insulin-mediated glucose uptake and vascular resistance, it would have been more straightforward to measure insulin-mediated microvascular recruitment in muscle instead of skin. However, the human skin is the only site available in humans to directly and to non-invasively assess capillary density and recruitment of capillaries. Moreover, the effects of obesity and free fatty acids on insulin-mediated microvascular recruitment in muscle [31, 32] can be reproduced in human skin [15, 33], suggesting that the vascular responses observed in skin reflect those in muscle. In a recent study, we could demonstrate concurrent insulin-mediated microvascular recruitment in skin and skeletal muscle. Both were mutually related and strongly associated with whole-body
glucose uptake suggesting that the cutaneous microcirculation is a representative vascular bed to examine insulin’s actions on the microcirculation [34].

In order to study underlying mechanisms of prednisolone-induced vascular insulin resistance, Wistar rats were additionally treated with prednisolone. Also in rats, insulin dose-dependently increased the diameter of isolated cremaster resistance arterioles, which was impaired by prednisolone treatment on all insulin levels. At the molecular level, prednisolone attenuated the vascular effects of insulin by reducing phosphorylation of the Akt pathway. Phosphorylation of the ERK1/2 pathway was not affected. Thus, by reducing Akt phosphorylation, prednisolone impaired insulin’s vasodilatory actions. Unfortunately, the amount of eNOS protein in one cremaster resistance artery is not sufficient to measure eNOS phosphorylation, but Akt phosphorylation has been shown to be a reliable marker of eNOS phosphorylation in this model [21, 35].

Interestingly, prednisolone treatment specifically attenuated the vasodilatory effects of insulin, since endothelium-dependent vasodilation as assessed by acetylcholine administration remained intact. This may indicate that specifically vascular insulin signaling is disturbed, whereas eNOS function is intact. Nevertheless, acetylcholine may also induce vasodilation by additional mechanisms than NO production, i.e. by releasing prostanoids and other endothelium-derived hyperpolarizing factors [36].

In conclusion, prednisolone treatment in healthy volunteers dose-dependently impaired insulin-stimulated capillary recruitment. In addition, prednisolone-induced changes in insulin-stimulated capillary recruitment were strongly related to unfavorable metabolic effects induced by prednisolone treatment, including impaired glucose tolerance, decreased insulin sensitivity as well as increased systolic blood pressure. Importantly, a substantial part of the decrease in insulin-stimulated glucose disposal could be explained by prednisolone-induced microvascular dysfunction. In isolated rat cremaster resistance arteries, we found evidence that prednisolone specifically impaired insulin-induced vasodilation by attenuating insulin-stimulated phosphorylation of the Akt pathway. Thus, we propose that vascular insulin resistance may contribute to the cardiovascular side effects associated with GC treatment.

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REFERENCES


SUPPLEMENTARY MATERIAL

Supplemental Figure 1. Study Design.

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</tbody>
</table>

S: Screening  
T1: Meal Tolerance Test  
T2: Euglycemic Clamp + Capillary  
M: study medication  
V: Follow up Visit

Supplemental Figure 2. Patient flow diagram. 100% of the randomized participants completed the study.
**Supplemental Figure 3.** Prednisolone treatment did not affect endothelium-dependent vasodilation as assessed by acetylcholine administration. A dose-response curve is presented. Data are mean ± SD. Significance was tested by Mann-Whitney U test. Prednisolone-treated rats, n=6; Saline-treated rats, n=6. Ach: acetylcholine.

**General characteristics of isolated cremaster arterioles following a 7-day treatment with prednisolone or saline.**

<table>
<thead>
<tr>
<th>General Characteristics</th>
<th>Prednisolone 8 mg/kg</th>
<th>Saline</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>A. Passive Diameter (mm)</td>
<td>184±14</td>
<td>173±8</td>
<td>0.180</td>
</tr>
<tr>
<td>B. Basal Diameter (mm)</td>
<td>75±25</td>
<td>83±19</td>
<td>0.485</td>
</tr>
<tr>
<td>C. Basal Tone (%)</td>
<td>60±11</td>
<td>52±12</td>
<td>0.310</td>
</tr>
<tr>
<td>D. Diameter change after Ach (%)</td>
<td>85±16</td>
<td>65±28</td>
<td>0.310</td>
</tr>
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</table>

Data are mean ± SD. Passive diameter was measured after isolation of cremaster arterioles. The basal diameter was measured at a pressure of 65 mmHg and 37°C. Basal arterial tone was calculated by \{[(A-B)/A]*100\}. Significance was tested by Mann-Whitney U test. Prednisolone-treated rats, n=6; Saline-treated rats, n=6. Ach: acetylcholine.