Chapter 5

Islet-cell Dysfunction Induced by Glucocorticoid Treatment: Potential Role for Altered Sympathovagal Balance?

ABSTRACT

Objectives: To assess the effects of low- and high-dose glucocorticoid (GC) treatment on pancreatic islet-cell function in healthy males and to study the potential influence of altered autonomous nervous system (ANS) balance and meal-related incretin responses.

Research design and methods: In a randomized, placebo-controlled, double-blind, dose-response intervention study, 32 males (age: 21±2 years; BMI: 21.9±1.7 kg/m²) were allocated to prednisolone 7.5 mg once daily (n=12), prednisolone 30 mg once daily (n=12), or placebo (n=8) for two weeks using block randomization. Main outcome measures were:

1) C-peptide secretion during a hyperglycemic clamp with additional arginine stimulation
2) fasting and postprandial glucagon levels
3) parasympathetic activity and sympathovagal balance
4) postprandial incretin responses.

Results: Prednisolone treatment did not significantly affect first- and second-phase glucose-stimulated C-peptide secretion, however, C-peptide secretion following arginine stimulation on top of hyperglycemia (ASI-iAUCp) was dose-dependently reduced: -2.8 (-5.2; -0.2) and -3.1 (-8.8; -1.0) nmol/L.min for prednisolone 7.5 mg and prednisolone 30 mg, respectively (P=0.035 vs. placebo). Fasting glucagon levels and postprandial glucagon levels were only increased by prednisolone 30 mg. Measures of the ANS and incretin responses were not significantly altered by treatment. However, changes in parasympathetic activity associated with fasting plasma glucose levels (r=-0.407; P=0.03) and tended to associate with fasting glucagon concentrations (r=-0.337; P=0.07). The change in sympathovagal balance was inversely related to ASI-iAUCp (r=-0.365; P=0.05). No side effects of prednisolone treatment were reported.

Conclusions: Impairments in islet-cell function contribute to prednisolone-induced glucose intolerance in healthy men. Altered sympathovagal balance may contribute to these effects.

Clinical Trial Registration Number: ISRCTN78149983
Glucocorticoids (GCs) are the cornerstone in the treatment of numerous diseases due to their potent anti-inflammatory and immunosuppressive actions [1]. However, pharmacological GC levels also induce adverse effects on glucose metabolism [1, 2]. In population-based studies, GC therapy was associated with incident diabetes [3]. Classically, the association between GCs and diabetes has been attributed to GC-induced insulin resistance [4].

The extent to which pancreatic islet-cell dysfunction, and particularly beta-cell dysfunction, contributes to the diabetogenic effects of GCs is less well-known. In vitro, GCs were shown to decrease insulin secretion and insulin synthesis [2]. In addition to reducing glucose-stimulated insulin secretion (GSIS), GCs impaired the in vitro effects of nonmetabolizable insulin secretagogues, including arginine and acetylcholine, suggesting that the site of action of GCs is in the end of the insulin secretory process [5]. Transgenic mice with a beta-cell specific overexpression of the glucocorticoid receptor (GR) develop diabetes due to beta-cell failure, in the presence of increased α-2 adrenergic activity [6].

In humans, a single high-dose of prednisolone was shown to impair both first-phase glucose-stimulated and arginine-stimulated C-peptide secretion during a hyperglycemic clamp [7] and glucose sensitivity of the beta cell during a meal challenge test [8]. (Sub)acute, GC-exposure (a 2 day-treatment), however, generated seemingly opposing results. A number of studies reported increased insulin secretion during a hyperglycemic clamp or intravenous glucose tolerance test following this treatment duration with high-dose GCs [9-13]. This most likely can be attributed to reduced insulin sensitivity. Only one of these studies measured insulin sensitivity with the hyperinsulinemic-euglycemic clamp [11], allowing adjustment prevailing insulin sensitivity. This study indicated impaired compensation in several subjects.

In addition to beta-cell function, GCs may also affect pancreatic alpha-cell function. Two studies showed increased glucagon secretion during high-dose GC treatment [14, 15].

However, there are no data available regarding the effects of more prolonged GC treatment on islet-cell function in humans. Also, the dose-dependency of these effects is largely unknown. Finally, mechanisms that could contribute to GC-induced effects on islet cell function in humans have, to our knowledge, not been investigated. Given the preclinical data [5, 6], alterations in the autonomic nervous system (ANS) balance could be implicated in GC-induced islet effects. Whereas parasympathetic branches of the ANS are well-known to stimulate insulin secretion via acetylcholine signaling [16], sympathetic fibers decrease insulin release via catecholamine-related pathways [17] and stimulate glucagon release [18]. Also, it is at present unclear whether the incretin hormones, important regulators of postprandial islet-cell function, may be involved in GC-induced islet effects.
Therefore, in the present study, we assessed the effects of a prolonged treatment, i.e. two weeks, with low or high-dose prednisolone, the most commonly prescribed GC, on islet-cell function in healthy normoglycemic males and cardiovascular ANS balance and meal-related incretin responses.

RESEARCH DESIGN AND METHODS

Participants: Thirty-two healthy Caucasian males were recruited by local advertisement. Inclusion criteria included: age 18-35 years, body mass index (BMI) 20.0-25.0 kg/m², 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-h glucose < 7.8 mmol/L following a 75g oral glucose tolerance test (OGTT), performed at 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 GC use, excessive sport activities (i.e. > 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 (n=8) treatment for a period of 14 days using block randomization, as carried out by the Department of Experimental Pharmacology of the VU University Medical Center. An outline of the study design is presented in Supplementary Figure 1. At baseline and day 13 of treatment, insulin sensitivity and beta-cell function were measured in a combined euglycemic-hyperglycemic clamp procedure (Supplementary Figure 2A). At baseline and day 14 of treatment, a standardized consecutive meal challenge test was performed and cardiovascular ANS function was measured in the fasted state (Supplementary Figure 2B). All measurements were conducted following a 12-h overnight fast with the subjects in the semi-supine position. Subjects refrained from drinking alcohol for a period of 24h before the study days and did not perform strenuous exercise for a period of 48h before the study days. During all visits, including a follow-up visit at day 7 of treatment, safety and tolerability were assessed. A patient flow diagram is shown in Supplementary Figure 3.
Hyperinsulinemic-euglycemic clamp and hyperglycemic clamp: After an overnight fast, participants were admitted to the clinical research unit at 7.30 AM. An indwelling cannula was inserted into an antecubital vein for infusion of glucose and insulin. To obtain arterialized venous blood samples, a retrograde cannula was inserted in a contralateral wrist vein and the hand placed in a heated box, maintained at 50°C. A primed, continuous (40 mU/m².min) insulin infusion (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was given for 120 min; plasma glucose was kept at 5 mmol/L by a variable infusion of 20% glucose, as described previously [19]. The hyperglycemic clamp was started 60 min after cessation of exogenous insulin infusion. Plasma glucose concentration was then raised to 10 mmol/L by a body weight-adjusted intravenous bolus of 20% glucose and a variable 20% glucose infusion was adjusted to maintain the targeted glucose level. After 80 min hyperglycemia, an intravenous bolus of 5 g arginine (dissolved in 50 mL NaCl) was given over 45 seconds, and the glucose level was maintained at 10 mmol/L for an additional 30 min (Supplementary Figure 2A).

Standardized consecutive meal challenge: After an overnight fast, participants were admitted to the clinical research unit at 7.30 AM. An indwelling cannula was inserted to allow blood sampling during the test. Two consecutive identical meals were served as breakfast at 09.00 AM and as lunch at 1.00 PM, each containing 905 kcal (50 g fat, 75 g carbohydrates, 35 g protein). Samples for determination of glucose, insulin, C-peptide, glucagon, glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP) were obtained at times 0, 5, 10, 20, 30, 60, 90, 120, 150, 180, 210 and 240 min following each meal, with the meal beginning immediately after the time 0 sample and being consumed within 15 min (Supplementary Figure 2B).

Heart rate variability: Prior to consumption of the first meal, continuous finger arterial blood pressure (BP) and heart rate (HR) were recorded (Portapres, FMS, Amsterdam, The Netherlands) in the supine position for 30 min on a beat-to-beat basis as described previously [20]. From the arterial pressure signal, interbeat interval was derived (Beatscope software 1.1, FMS, Amsterdam, The Netherlands). Power spectral analysis was assessed by discrete Fourier transform as described previously [21]. The low-frequency band (LF; 0.04-0.15 Hz) and the high frequency band (HF; 0.15-0.4 Hz) were selected. LF and HF bands were expressed in normalized units (LF\_norm and HF\_norm, respectively), where HF\_norm represents parasympathetic activity. In addition, the LF/HF ratio was computed as a measure of sympathovagal balance [21].

Study medication: Prednisolone tablets were purchased from Pfizer AB (Sollentuna, Sweden) and placebo tablets were obtained from Xendo Drug Development (Groningen, The Netherlands).
Netherlands). Tablets were capsulated in order to allow the treatment to be blinded [8]. 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.

**Analytical determinations:** Blood glucose concentrations were measured using an YSI 2300 STAT Plus analyzer (YSI, Yellow Springs, OH). Insulin and C-peptide levels were determined using an immunometric assay (Advia, Centaur, Siemens Medical Solutions Diagnostics, USA). Glucagon concentrations were determined by radioimmunoassay (Linco Research, St. Louis, USA). Total GLP-1 levels were measured using a C-terminally directed radioimmunoassay (antiserum no. 89390) as described previously [22]. Total GIP was analyzed using a newly developed assay, employing a C-terminally directed antiserum (code no 80867) raised in rabbits immunized with a C-terminal fragment of GIP [GIP (28-42)] conjugated to keyhole limpet haemocyanin via its N-terminus. This assay has broadly the same specificity and characteristics as the previously published assay using antiserum R65 [23], recognizing equally both intact GIP (1-42) and the primary metabolite, GIP (3-42).

**Data analyses:** From the hyperglycemic clamp the beta-cell function parameters first-phase (min 0-10) and second-phase (min 10-80) incremental area under the C-peptide curve (iAUC\textsubscript{CP}) as well as arginine-stimulated C-peptide secretion (ASI-iAUC\textsubscript{CP}) (min 80-110) were calculated using the trapezoid method (nmol/L.min). From the hyperinsulinemic-euglycemic clamp 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 [19]. During the double meal challenge absolute area under the curves (AUC\textsubscript{s}) for glucose, insulin, C-peptide, glucagon, GLP-1 and GIP were calculated using the trapezoid method.

**Beta-cell function during the meal challenge test:** Beta-cell function during the standardized double meal challenge test was assessed by mathematical modeling which was described in detail previously [24]. The model describes the relationship between insulin secretion and glucose concentration as the sum of two components. The first component represents the dependence of insulin secretion on absolute glucose concentrations at any time point and is characterized by a dose-response function relating the two variables. The characteristic parameter of the dose response is its mean slope, denoted here as glucose sensitivity. The dose response is modulated by both glucose-mediated and non-glucose-mediated factors (i.e. non-glucose substrates, gastrointestinal hormones, and neurotransmitters), which are collectively modeled as a potentiation factor. The excursion of the potentiation factor was quantified using a ratio between mean values at times 160-180 min and 0-20 min, and is
called the *potentiation factor ratio* (PFR). In addition, the *fasting secretory tone* is calculated from the dose-response curve as insulin secretion at the glucose concentration of 4.5 mmol/L. The second component of the model describes the insulin response to the rate of change of glucose concentration. This component is termed *rate sensitivity*, which is related to early insulin release [24].

**Statistical analyses:** Data are presented as mean values ± standard deviation (SD), or as median (interquartile range) in case of skewed distribution. Non-parametric analysis was chosen due to uneven sample size over the groups, the relatively small N, and unequal variances that were observed for some parameters. Between-group comparisons of baseline values were performed using Kruskal-Wallis test. For treatment-induced effects, absolute changes from baseline were calculated (on-treatment value minus pre-treatment value) and were compared by Kruskal-Wallis test with trend analysis ('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 Pearson’s correlations. All statistical analyses were run on SPSS version 15 for Windows (Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

**RESULTS**

**Anthropometric characteristics:** No significant differences in subject characteristics were observed among the groups at baseline (Table 1). Body weight was not altered by prednisolone-treatment irrespective of the dose (Supplementary Table 1). Systolic blood pressure (6±1.2 mmHg increase; $P=0.006$), but not diastolic blood pressure was raised by prednisolone 30 mg. Prednisolone 7.5 mg did not affect blood pressure (Supplementary Table 1).

**Fasting glucose and hormone levels:** A dose-dependent rise in fasting plasma glucose levels was observed by prednisolone treatment relative to placebo ($P=0.04$) (Table 2). Fasting plasma insulin levels were significantly increased ($P=0.008$) in the prednisolone 30 mg arm. The Prednisolone-induced increase in fasting insulin levels was due to increased basal secretion, not altered clearance, since fasting C-peptide levels were similarly enhanced ($P=0.001$) (Table 2).
Table 1. Subject characteristics at inclusion

<table>
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<tr>
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<th>Placebo</th>
<th>Prednisolone</th>
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<td>30 mg</td>
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</tr>
<tr>
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<tr>
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<td>185±5</td>
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<td>120±9</td>
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<td>Diastolic blood pressure (mmHg)</td>
<td>79±10</td>
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<td>78±10</td>
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<td>Fasting plasma glucose (mmol/l)</td>
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<td>4.5±0.2</td>
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<td>2-hr glucose OGTT (mmol/l)</td>
<td>3.7±1.0</td>
<td>3.9±0.9</td>
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</table>

Data are mean±SD. 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.

Table 2. Fasting metabolic parameters before and on day 14 of treatment with placebo, prednisolone 7.5 mg once daily and prednisolone 30 mg once daily.

<table>
<thead>
<tr>
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<th>P2</th>
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<td>PLB</td>
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<td>Fasting plasma glucose (mmol/l)</td>
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<td>0.418</td>
<td>0.09</td>
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<td>7.5</td>
<td>4.4±0.2</td>
<td>4.7±0.3</td>
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<td>1.0</td>
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<tr>
<td></td>
<td>30</td>
<td>4.5±0.2</td>
<td>4.9±0.4</td>
<td>0.001</td>
<td>0.918</td>
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<td></td>
<td>30</td>
<td>32 (26-44)</td>
<td>56 (41-72)</td>
<td>0.001</td>
<td>0.152</td>
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<tr>
<td>Fasting plasma insulin (pmol/l)</td>
<td>31 (28-40)</td>
<td>33 (28-39)</td>
<td>0.001</td>
<td>1.0</td>
<td>0.008</td>
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<tr>
<td></td>
<td>7.5</td>
<td>36 (29-47)</td>
<td>36 (27-65)</td>
<td>0.001</td>
<td>0.918</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>32 (26-44)</td>
<td>56 (41-72)</td>
<td>0.001</td>
<td>0.152</td>
</tr>
<tr>
<td>Fasting C-peptide (nmol/l)</td>
<td>0.4±0.1</td>
<td>0.4±0.1</td>
<td>0.5±0.2</td>
<td>0.001</td>
<td>0.918</td>
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<tr>
<td></td>
<td>7.5</td>
<td>0.4±0.1</td>
<td>0.5±0.2</td>
<td>0.001</td>
<td>0.918</td>
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<td>30</td>
<td>0.4±0.1</td>
<td>0.6±0.2</td>
<td>0.001</td>
<td>0.918</td>
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<tr>
<td>Fasting glucagon (pmol/l)</td>
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<td>10±3</td>
<td>0.002</td>
<td>0.152</td>
<td>0.014</td>
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<td>12±3</td>
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<tr>
<td></td>
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<td>12±3</td>
<td>15±3</td>
<td>0.002</td>
<td>0.152</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, posthoc testing (by Mann-Whitney U) was done (P2 and P3). Treatment groups: PLB=placebo; 7.5=prednisolone 7.5 mg daily; 30=prednisolone 30 mg daily.

Hyperglycemic clamp: Prednisolone treatment tended to increase first- and second-phase glucose-stimulated C-peptide secretion (Figure 1A-B). In multivariate analysis however, this trend was no longer observed when the M-value was added in the model (1st-phase...
iAUC: $\beta=0.126; P=0.638; R^2=0.119$ and 2nd-phase iAUC: $\beta=0.051; P=0.847; R^2=0.152$.

C-peptide secretion following arginine stimulation on top of hyperglycemia ($ASi$-iAUC$_{cp}$) was significantly and dose-dependently reduced by prednisolone treatment: -2.7 (-5.2; -0.3) nmol/L.min for prednisolone 7.5 mg and -3.0 (-7.6; -0.2) nmol/L.min for prednisolone 30 mg ($P=0.035$) (Figure 1C). In multivariate analysis, this relation was independent of changes in insulin sensitivity. The results from the hyperglycemic clamp were similar when insulin iAUCs were calculated (data not shown).

Euglycemic clamp: Prednisolone treatment dose-dependently decreased insulin sensitivity ($M$-value) as compared to placebo: mean differences: -2.1 ± 0.8 mg kg$^{-1}$ min$^{-1}$ for prednisolone 7.5 mg and -4.5 ± 0.7 mg kg$^{-1}$ min$^{-1}$ for prednisolone 30 mg (Figure 2). Insulin levels reached steady state during min 90-120 of the euglycemic clamp at 490 ± 77 pmol/L prior to treatment, and were not altered during the on-treatment clamps (Supplementary Table 2). Adjustment of the $M$-value by insulin levels during the steady-state part of the clamp ($M/I$) did not affect the results.

**Figure 1.** The effects of prednisolone treatment on first-phase (A) and second-phase (B) glucose-stimulated, and arginine-stimulated C-peptide secretion (C) during the hyperglycemic clamp. Prednisolone 30 mg tended to increase first- and second-phase C-peptide secretion. However, both dosages of prednisolone significantly decreased C-peptide secretion following arginine administration on top of hyperglycemia. Box-and-Whisker plots (min-max) are shown. White bars: before treatment; black bar: on treatment. Between-group changes from baseline were tested by Kruskal-Wallis test 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). *$P <0.05$; † $P=0.074$; ‡ $P=0.09$.

Standardized double meal challenge test: Prednisolone treatment dose-dependently increased AUC for glucose (AUC$_G$) by 11±5% ($P=0.008$) and 27±9% ($P<0.001$), for prednisolone 7.5 mg and prednisolone 30 mg respectively (Figure 3A), despite dose-dependent increases in postprandial insulin and C-peptide levels (Figure 3B-C). Plasma levels of total GLP-1 and GIP were not changed by either of the prednisolone dosages (Figure 3D-E).

**Beta-cell function during the standardized double meal challenge test:** Neither prednisolone 7.5 mg nor prednisolone 30 mg affected beta-cell glucose sensitivity and rate
sensitivity (Figure 4A-B). PFR was significantly decreased by prednisolone 30 mg, but not by prednisolone 7.5 mg (Figure 4C). Basal insulin secretion at a fixed glucose level of 4.5 mmol/L tended to decrease following prednisolone treatment (P=0.052) (Figure 4D).

**Figure 2.** Prednisolone treatment dose-dependently decreased insulin sensitivity during the hyperinsulinemic-euglycemic clamp. Box-and-Whisker plots with the absolute change from baseline are shown. Between-group changes from baseline were tested by Kruskal-Wallis test 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=0.062.

Glucagon levels: Fasting glucagon levels were increased by prednisolone 30 mg treatment, but not by prednisolone 7.5 mg (Table 2). Similarly, only prednisolone 30 mg (P=0.004) increased postprandial glucagon levels (Figure 3F).

Heart rate variability: Before treatment, LF/HF ratio in the entire study population was 1.42 (0.86-1.85) arbitrary units (AU), LF$_{norm}$ 42 (27-53) AU, and HF$_{norm}$ 33 (22-41) AU. Prednisolone treatment did not affect LF/HF ratio, but prednisolone 30 mg treatment non-significantly decreased HF$_{norm}$ by 14±5 AU as compared to placebo (P=0.06).

Associations between heart rate variability and islet-cell function: The change in HF$_{norm}$ was inversely associated with changes in fasting plasma glucose levels (Figure 5A) and tended to negatively associate with fasting glucagon levels ($\beta$=-0.337; P=0.07). The change in LH/FH ratio was inversely related with changes in ASI-iAUC$_{cp}$ ($\beta$=-3.65; P=0.05) (Figure 5B). There were no significant associations between measures of HRV and postprandial glucagon levels or measures of beta-cell function obtained during the meal test (data not shown).
Figure 3. Postprandial responses at baseline and at 14 day of intervention. Panel A: Glucose concentrations. Panel B: Insulin concentrations. Panel C: C-peptide concentrations. Panel D: Glucagon-like peptide (GLP)-1 concentrations. Panel E: Glucagon concentrations. Panel F: Glucagon concentrations. Straight line with black squares denotes pre-treatment. Dotted line with open circles represents on-treatment. Box-and-Whisker plots with absolute change in area under the curve (AUC) from baseline are shown. Between-group changes from baseline were tested by Kruskal-Wallis test 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 4. Results from the modeling analysis of beta-cell function from glucose and C-peptide levels during 2 consecutive standardized meals given as breakfast (t=0) and as lunch (t=240 min). Glucose sensitivity of the beta cell (A) and rate sensitivity (B) were not altered by study medication. Potentiation factor ratio between T=160-180 min and T=0-20 min was significantly decreased by prednisolone 30 mg (C). Finally, prednisolone treatment non-significantly reduced fasting insulin secretion rates at a fixed glucose level of 4.5 mmol/L. Box-and-Whisker plots (min-max) are shown. White bars: before treatment; black bar: on treatment. Between-group changes from baseline were tested by Kruskal-Wallis test 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 <0.01; *P<0.05; # P=0.052.

Figure 5. Associations between changes from baseline in HFnorm and changes from baseline changes in FPG (A) and changes from baseline in LH/FH ratio and changes from baseline in arginine-stimulated C-peptide secretion (B). Associations were tested with Pearson correlations.
Safety and tolerability: 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 any of the treatment arms.

DISCUSSION

The principal findings of the present study are that a two-week treatment with high-dose prednisolone impaired various parameters of pancreatic islet-cell function, and that altered ANS balance may be involved in these changes, although ANS balance itself was not significantly altered. Furthermore in this study, we observed a clear dose-dependency of prednisolone-induced effects. However, probably due to lack of power, the effects of low-dose prednisolone treatment did not reach statistical significance. At increased levels, GCs induce glucose intolerance and diabetes in susceptible individuals, which has classically been attributed to GC-induced insulin resistance (4). Indeed, our study confirmed the presence of GC-induced insulin resistance: prednisolone treatment dose-dependently reduced clamp-measured M-value.

Figure 6. The relation between treatment-induced changes in insulin sensitivity and 1st phase glucose-stimulated C-peptide secretion for placebo (A), prednisolone 7.5 mg daily (B) and prednisolone 30 mg daily (C). As demonstrated in (C), during treatment with prednisolone 30 mg, C-peptide secretion is enhanced to compensate for reduced insulin sensitivity. Black squares: pre-treatment. Open circles: on-treatment. Grey triangle: mean pre-treatment. Open triangle: mean on-treatment.

The effects of short-term GC treatment on islet-cell function, and particularly beta-cell function, have been under debate. The observation that 2 days GC exposure induced fasting hyperinsulinemia and increased insulin secretion in response to oral and intravenous glucose loads, may suggest a lowering of insulin sensitivity by GCs [9-13]. In the present study, we similarly observed elevated fasting insulin levels, postprandial hyperinsulinemia and a tendency towards increased first- and second-phase glucose-stimulated C-peptide secretion. Using multivariate analysis, we demonstrated in this study that increased 1st and 2nd phase
C-peptide secretion was driven by treatment-related reduction in insulin sensitivity, showing adequate compensation for prednisolone-induced insulin resistance (Figure 6), and did not indicate improved beta-cell function per se. Interestingly, prednisolone treatment reduced arginine-stimulated C-peptide secretion, a measure of insulin secretory capacity, in a dose-dependent manner.

A similar pattern was observed during the meal challenge test. Modeling analysis of glucose and C-peptide concentrations, revealed decreased glucose-adjusted insulin secretion rates following prednisolone treatment, also at fasting glucose levels (Supplementary Figure 4; Figure 4D for insulin secretion at 4.5 mmol/L glucose). While prednisolone treatment did not affect glucose sensitivity of the beta-cell or rate sensitivity, prednisolone 30 mg markedly decreased the potentiation factor ratio, a finding that confirms data from a previous study published by our group [8]. This represents insulin secretion that is not primarily related to plasma glucose levels and may include secretion induced by non-glucose secretagogues, incretin hormones, and neuronal factors [25, 26].

From both the hyperglycemic clamp test and the meal test we conclude that prednisolone induces beta-cell dysfunction, although this effect is detectable on specific beta-cell function parameters that seem to involve potentiation phenomena. Both arginine-induced secretion at 10 mmol/L glucose [27] and potentiation factor ratio [24, 26] during the meal are dependent on potentiation. On the other hand, the enhancement of first- and second phase insulin secretion as well as postprandial insulin release is mainly mediated by an increase in glucose levels and a compensatory response to prednisolone-induced insulin resistance. In addition to impairments in beta-cell function, we observed GC-induced increased fasting and postprandial glucagon levels during high-dose prednisolone treatment. Thus, prednisolone treatment altered islet-cell functional balance.

Additionally, we evaluated mechanisms possibly underlying the effects of GCs on islet-cell function. First, we evaluated the role of the ANS system. Whereas catecholamines released by the sympathetic nervous system inhibit insulin secretion via $\alpha_2$-adrenergic receptor (AR) signaling and stimulate glucagon release, acetylcholine released by parasympathetic nerves stimulates insulin release via protein kinase C-related pathways [2]. In addition, parasympathetic nerve fibers increase islet blood flow, thus facilitating increased insulin secretion [28]. In beta-cell lines, GCs decreased the efficacy of acetylcholine to release insulin [5], and also upregulated expression and signaling of $\alpha_2$-ARs [29]. Transgenic mice with beta-cell specific overexpression of the GR were shown to develop diabetes through beta-cell failure consequential to increased $\alpha_2$-AR expression. We observed a tendency
towards withdrawal of vagal activity following high-dose prednisolone treatment, which was negatively associated with fasting glucose and glucagon levels. In addition, alterations in sympathovagal balance, expressed as LF/HF ratio, were negatively related to changes in C-peptide secretion in response to arginine. The latter finding is partly in line with a previous study, in which interruption of cholinergic transmission by trimethaphan impaired arginine-stimulated insulin secretion during treatment with dexamethasone [30]. However, in that acute study, a 2-day treatment with high-dose dexamethasone, increased arginine-stimulated secretion, whereas we observed a decline in this beta-cell function parameter. Differences in treatment duration and study population (in that study, highly insulin sensitivity participants, as determined by hyperinsulinemic-euglycemic clamp prior to inclusion, were studied) may have contributed to these seemingly opposing results. In addition, dexamethasone is a pure GR agonist, whereas prednisolone also activates the mineralocorticoid receptor. Various GC compounds, depending on their receptor specificity, may have a different effect on measures of cardiovascular autonomic function and variables such as blood pressure and heart rate, making the compounds difficult to compare [31, 32].

Second, we measured incretin responses following prednisolone treatment. The incretin hormones GLP-1 and GIP substantially contribute to glucose tolerance and we hypothesized that prednisolone treatment would reduce incretin levels [33]. However, we did not observe declined plasma levels of GLP-1 and GIP following prednisolone treatment, confirming a recently published study, in which high-dose prednisolone treatment combined with a hypercaloric diet and physical inactivity did not decrease postprandial levels of GLP-1 and GIP [14]. However, in the same study, prednisolone treatment was shown to reduce the insulinotropic effects of GLP-1 and GIP. Thus, an impaired incretin effect at the level of the beta cell may in fact contribute to GC-induced hyperglycemia. Future in vitro studies will need to address this hypothesis.

The observations done in the present study may have important implications for the treatment of GC-induced glucose intolerance and diabetes. Our data indicate that pharmacological measures that aim to improve pancreatic islet-cell function may particularly be effective in restoring glucose tolerance during prednisolone treatment. In line with this hypothesis, we have recently shown that the acute effects of high-dose prednisolone treatment on glucose tolerance and islet-cell function in healthy humans could be prevented by concomitant treatment with the GLP-1 receptor agonist exenatide [7]. Further studies in relevant populations should explore the full potential of incretin-based therapies to prevent GC-induced glucose intolerance.
We conclude that, in addition to reducing insulin sensitivity, prolonged prednisolone treatment dose-dependently impairs islet-cell function in healthy males. Our data furthermore suggest that changes in ANS balance may contribute to these GC-related changes.

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REFERENCES


SUPPLEMENTARY FIGURES

Supplementary Figure 1. Study Design.

Supplementary Figure 2A. Combined Hyperinsulinemic-Euglycemic and Hyperglycemic Clamp Procedure.

Supplementary Figure 2B. Double Standardized Meal Challenge Test.
**Supplementary Figure 3.** Patient flow diagram. 100% of the randomized participants completed the study.

**Supplementary Figure 4.** Dose-response curve for insulin secretion and glucose levels during the meal challenge test. The dose-response curve is shifted downwards by both PRED dosages. Panel A: Placebo. Panel B: Prednisolone 7.5 mg daily. Panel C: Prednisolone 30 mg daily. Straight line with black squares denotes pre-treatment. Dotted line with open circles represents on-treatment.
### Supplementary Table 1. Body weight and blood pressure before and during two-week treatment with placebo, prednisolone 7.5 mg once daily, or prednisolone 30 mg once daily.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>On-treatment</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
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<tbody>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>PLB 75±8</td>
<td>76±7</td>
<td>0.539</td>
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<td></td>
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<td>74±10</td>
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<td><strong>BMI (kg/m²)</strong></td>
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<td>21.7±1.3</td>
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<td>NA</td>
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<tr>
<td></td>
<td>7.5 22.1±1.5</td>
<td>22.1±1.7</td>
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<tr>
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<tr>
<td><strong>Systolic Blood Pressure (mmHg)</strong></td>
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<td>0.710</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>30  120±9</td>
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<td><strong>Diastolic Blood Pressure (mmHg)</strong></td>
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<td>30  78±10</td>
<td>80±9</td>
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</tbody>
</table>

Means±SD or median (interquartile range) are provided. 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 and P3). Treatment groups: PLB=placebo; 7.5=prednisolone 7.5 mg daily; 30=prednisolone 30 mg daily.

### Supplementary Table 2. Clamp insulin levels before and during two-week treatment with placebo, prednisolone 7.5 mg once daily, or prednisolone 30 mg once daily.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>On-treatment</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clamp Insulin levels (pmol/l)</strong></td>
<td>PLB 441±81</td>
<td>412±49</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>7.5 488±61</td>
<td>453±69</td>
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<tr>
<td></td>
<td>30  504±78</td>
<td>460±81</td>
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Means±SD or median (interquartile range) are provided. Between-group changes from baseline were tested by Kruskal-Wallis with trend analysis (P). Treatment groups: PLB=placebo; 7.5=prednisolone 7.5 mg daily; 30=prednisolone 30 mg daily.