Chronic Prednisolone Treatment Aggravates Hyperglycemia in Mice Fed a High-Fat Diet but Does Not Worsen Dietary Fat-Induced Insulin Resistance

Laskewitz, Anke J.; van Dijk, Theo H.; Grefhorst, Aldo; van Lierop, Marie-Jose; Bijker-Schreurs, Marijke; Bloks, Vincent; Reijngoud, Dirk; Dokter, Wim H.; Kuipers, Folkert; Groen, Albert

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Chronic Prednisolone Treatment Aggravates Hyperglycemia in Mice Fed a High-Fat Diet but Does Not Worsen Dietary Fat-Induced Insulin Resistance


Departments of Pediatrics (A.J.L., V.W.B., F.K., A.K.G.) and Laboratory Medicine (T.H.v.D., D.-J.R., F.K.) and Pathology and Medical Biology and Molecular Genetics (M.S.), University of Groningen, University Medical Center Groningen, 9700 RB, Groningen, The Netherlands; Department of Internal Medicine (A.G.), Erasmus Medical Center, 3000 CA Rotterdam, The Netherlands; Immunotherapeutics (M.-J.v.L.), MSD, 5340 BH Oss, The Netherlands; and Synthon Research Laboratories (W.H.D.), 6545 CM Nijmegen, The Netherlands

Synthetic glucocorticoids such as prednisolone have potent antiinflammatory actions. Unfortunately, these drugs induce severe adverse effects in patients, many of which resemble features of the metabolic syndrome, such as insulin resistance. In this study, we investigated whether adverse effects of prednisolone on glucose homeostasis are aggravated in mice with compromised insulin sensitivity due to a high-fat diet by applying various methods to analyze changes in insulin sensitivity in mice. C57BL/6J mice were fed a high-fat diet for 6 wk and treated with either prednisolone (10 mg/kg·d) or vehicle for the last 7 d. Insulin sensitivity and blood glucose kinetics were analyzed with state-of-the-art stable isotope procedures in different experimental conditions. Prednisolone treatment aggravated fasting hyperglycemia and hyperinsulinemia caused by high-fat feeding, resulting in a higher homeostatic assessment model of insulin resistance. In addition, prednisolone-treated high-fat diet-fed mice appeared less insulin sensitive by detailed analysis of basal glucose kinetics. Remarkably, using hyperinsulinemic-euglycemic or hyperglycemic clamp techniques, neither hepatic nor peripheral insulin resistance was worsened in the group that was treated with prednisolone. Yet analysis of hepatic glucose metabolism revealed that prednisolone did alter glycogen balance by reducing glycogen synthase flux under hyperinsulinemic as well as hyperglycemic conditions. In addition to elevated insulin levels, prednisolone-treated mice showed a major rise in plasma leptin and fibroblast growth factor 21 levels. Our data indicate that prednisolone-induced adverse effects on glucose metabolism in high-fat diet-fed mice do not reflect impaired insulin sensitivity but may be caused by other changes in the hormonal regulatory network controlling glucose metabolism such as fibroblast growth factor 21 and leptin. (Endocrinology 153: 3713–3723, 2012)

Glucocorticoids are steroid hormones produced by the adrenal gland under control of the hypothalamic-pituitary-adrenal axis. Cortisol and corticosterone are the major endogenous glucocorticoids in humans and rodents, respectively. Cortisol is regarded as a stress hormone and is involved in regulation of a plethora of processes that modulate blood pressure, salt and water balance, immune response, energy metabolism, and glucose homeostasis (1, 2). Related to their effects on the immune response, synthetic glucocorticoids, (e.g. prednisolone) have potent antiinflammatory actions. Unfortunately, these drugs induce severe adverse effects in patients, many of which resemble features of the metabolic syndrome, such as insulin resistance. In this study, we investigated whether adverse effects of prednisolone on glucose homeostasis are aggravated in mice with compromised insulin sensitivity due to a high-fat diet by applying various methods to analyze changes in insulin sensitivity in mice. C57BL/6J mice were fed a high-fat diet for 6 wk and treated with either prednisolone (10 mg/kg·d) or vehicle for the last 7 d. Insulin sensitivity and blood glucose kinetics were analyzed with state-of-the-art stable isotope procedures in different experimental conditions. Prednisolone treatment aggravated fasting hyperglycemia and hyperinsulinemia caused by high-fat feeding, resulting in a higher homeostatic assessment model of insulin resistance. In addition, prednisolone-treated high-fat diet-fed mice appeared less insulin sensitive by detailed analysis of basal glucose kinetics. Remarkably, using hyperinsulinemic-euglycemic or hyperglycemic clamp techniques, neither hepatic nor peripheral insulin resistance was worsened in the group that was treated with prednisolone. Yet analysis of hepatic glucose metabolism revealed that prednisolone did alter glycogen balance by reducing glycogen synthase flux under hyperinsulinemic as well as hyperglycemic conditions. In addition to elevated insulin levels, prednisolone-treated mice showed a major rise in plasma leptin and fibroblast growth factor 21 levels. Our data indicate that prednisolone-induced adverse effects on glucose metabolism in high-fat diet-fed mice do not reflect impaired insulin sensitivity but may be caused by other changes in the hormonal regulatory network controlling glucose metabolism such as fibroblast growth factor 21 and leptin. (Endocrinology 153: 3713–3723, 2012)
nisolone) are widely used as antiinflammatory and immuno-
suppressive drugs (3, 4). Unfortunately, chronic gluco-
corticoid treatment is accompanied by many adverse
effects in humans, among which are central obesity, hy-
pertension, hyperlipidemia, hyperglycemia, and insulin
resistance (5, 6). These adverse effects also represent com-
ponents of the metabolic syndrome, a condition defined by
a combination of symptoms that increase the risk of car-
diovascular disease and diabetes. Stress and elevated cor-
tisol levels have been reported to contribute to develop-
ment of the metabolic syndrome (7).

Induction of insulin resistance by prednisolone in hu-
mans was established by Pagano et al. in 1982 (8). A
more recent elegant study showed that prednisolone
readily induces insulin resistance in humans at a low
dose (9). To be able to overcome the undesired adverse
effects of glucocorticoids, the underlying mechanisms
of their modes of action must be resolved. Several stud-
ies to this end have been performed in rats, e.g. after
treatment with dexamethasone (10–13). Reported ef-
ects of glucocorticoids in conventional mice, however, are
highly variable. This is of relevance because genetically
modified mouse models offer great possibilities to dissect
molecular mechanisms of insulin resistance (14). Orland
and Permutt (15) showed strain-specific effects of dexa-
methasone in db/db mice on either a C57BL/KsJ or
C57BL/6J background, but 4 d dexamethasone treatment
did not affect blood glucose levels in the control mice of
either strain, whereas serum insulin levels were minimally
increased. Dexamethasone treatment increased plasma
blood glucose levels in female A^vy/a (viable yellow) mice,
but did not further increase plasma glucose levels in (hy-
perglycemic) male A^vy/a mice (16). In C57BL/6J mice, 5 d
of alternate dexamethasone treatment did not affect fast-
ing plasma glucose and insulin levels under conditions of
high-fat diet-induced obesity (17).

We have recently shown that chronic prednisolone
treatment for 7 d in C57BL/6J mice kept on a standard
laboratory chow does affect hepatic glucose production
(HGP) in the fasted state in a very subtle manner (18).
When the gold standard to determine insulin sensitivity,
i.e. the hyperinsulinemic-euglycemic clamp (HIEC), was
applied, we were not able to demonstrate effects of chronic
prednisolone treatment on insulin sensitivity. In fact, ap-
parent insulin resistance was detectable only under con-
ditions characterized by physiologically relevant insulin
concentrations. Feeding mice a high-fat diet compromises
insulin sensitivity (19), and we hypothesized that this more
closely resembles the human situation.

In the present study, we therefore investigated whether
prednisolone affects insulin sensitivity in mice fed a high-
fat diet (36% energy from lard) and whether we could
discern effects of prednisolone on hepatic glucose metab-
olism under these conditions. Altogether, our data indi-
cate that prednisolone alters hepatic glucose metabolism
by bringing about selective changes in the complex net-
work of hormones that control glucose metabolism.

Materials and Methods

Animals, treatments, and diets

Male C57BL/6J mice were obtained from Harlan (Horst, The
Netherlands) and housed under conventional conditions. Mice
were fed a high-fat diet (beef tallow, rich in saturated fat) (20) for
6 wk (Abdiets, Woerden, The Netherlands). After 5 wk of diet,
the mice received prednisolone (10 mg/kg · d) or vehicle [gelatin
(0.5 g/liter)/mannitol (5 g/liter)] daily by gavage at 1300 h for 7 d
(n = 7–8). All experiments were approved by the Ethics Com-
mmittee for Animal Experiments of the University of Groningen.
Prednisolone and vehicle were kindly provided by MSD (Oss,
The Netherlands). For the infusion studies, mice were equipped
with a permanent jugular vein catheter, with a two-way entrance
attached to the skull with acrylic glue as previously described
(21). Plasma levels of diabetes biomarkers were measured in fed
(at 2300 h) and 9-h fasted (from 2300–0800 h) mice.

Blood glucose kinetics

To determine blood glucose kinetics in mice multiple times
during the high-fat diet, mice were fasted for 9 h (2300–0800 h).
As previously described (22–24), we used a single-pool first-
order kinetics model because there is no dynamic insulin action
under these conditions. After the fasting period, the mice were
injected ip at the start of the experiment, after 5 wk and after 6
wk with a low dose of D-[6,6-2H2]glucose (100 mg/kg) without
inducing hyperinsulinemia. Before (at time zero) and at 10, 20,
30, 40, 50, 60, 75, and 90 min after injection, blood glucose
concentrations were measured in blood collected by tail tip
bleeding with a Lifescan EuroFlash (Lifescan Benelux, Beere,
Belgium) glucose meter. At the same time points, small blood
samples were collected on filter paper, in which the fractional
contribution of D-[6,6-2H2]glucose to the whole blood glucose
pool was measured by gas chromatography GC-mass spectrom-
etry (GC-MS). Whole-body glucose turnover and clearance were
calculated by kinetic analysis of the washout of injected D-[6,6-
2H2]glucose from the circulation (Supplemental Table 1, pub-
lished on The Endocrine Society’s Journals Online web site at
http://endo.endojournals.org). At the end, a blood sample was
taken by tail tip bleeding for insulin measurements.

Determination of hepatic carbohydrate fluxes

before and during HIEC and hyperglycemic clamp

(HGC)

For the HIEC and HGC, two separate groups of C57BL/6J
mice were operated for jugular vein catheterization at d 2 of the
treatment with prednisolone. After surgery, mice were allowed
to recover for 5 d before they were subjected to experiments,
resulting in 7 d of prednisolone treatment. Before the exper-
iments, mice were fasted for a 9-h (2300–0800 h) or a 4-h (0400–
0800 h) period for HIEC and HGC, respectively. During the

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experiment, mice were kept in small Plexiglas cages, with a wired floor to allow urine sampling and frequent collection of blood samples in freely moving mice (25). To determine hepatic carbohydrate fluxes under basal conditions, mice were infused for a 4-h basal period with a solution containing [U-13C]glucose (2.5 mg/ml), [2,13C]glycerol (15 mg/ml), [1-2H]galactose (6 mg/ml) (Cambridge Isotope Laboratories, Andover, MA), and paracetamol (1 mg/ml) at an infusion rate of 0.54 ml/h as previously described (25,26). For the HIEC, solutions were changed after 4 h, and mice were subjected to a 4-h hyperinsulinemic period by infusing a solution containing insulin (44 µU/ml, Actrapid; Novo Nordisk, Bagsvaerd, Denmark), somatostatin (40 µg/ml; UCB, Breda, The Netherlands), [2-13C]glycerol (60 mg/ml), [1-2H]galactose (24 mg/ml), paracetamol (4 mg/ml), and 1% BSA (Sigma, St. Louis, MO) at a constant rate of 0.135 ml/h. During hyperinsulinemia, euglycemia was kept by infusion of a second solution that contained 27% glucose (291 mg/ml) and 3% [U-13C]glucose (9 mg/ml) at an adjustable rate to maintain plasma glucose levels at 7 mM. For the second experiment, the HGC, infusions were changed after 4 h and mice were subjected to a 4-h hyperglycemic period by infusion of a solution containing [U-13C]glucose (10 mg/ml), [2,13C]glycerol (60 mg/ml), [1-2H]galactose (24 mg/ml), and paracetamol (4 mg/ml) and 1% BSA at a constant rate of 0.135 ml/h. A second solution that contained 27% glucose (291 mg/ml) and 3% [U-13C]glucose (9 mg/ml) was infused with an adjustable rate to maintain plasma glucose levels at a hyperglycemic level of approximately 17 mM, comparable to the peak blood glucose level measured in control mice during an oral glucose tolerance test. During both experiments, blood glucose levels were measured every 15 min in blood drops collected by tail tip bleeding with a Lifescan EuroFlash glucose meter. Every hour, blood spots for GC-MS analysis were taken by tail tip bleeding on filter paper, air dried, and stored at room temperature until further analysis. Urine samples were collected on filter paper at hourly intervals. Hepatic carbohydrate fluxes as well as whole-body glucose turnover and clearance rates during the experiment were calculated using mass isotopomer distribution analysis (MIDA) as previously described (26,27). Additionally, after the hyperglycemic period a blood sample was taken for insulin measurements.

Indirect calorimetry

Mice fed a high-fat diet for almost 5 wk were subjected to indirect calorimetry for 7 d with free access to water and a high-fat diet, whereas food intake was measured (TSE Systems GmbH, Bad Homburg, Germany). Three days before the start of the prednisolone treatment, mice were placed in the calorimetric cages to acclimatize and for basal measurement. Upon start of the treatment, mice were tracked for the 4 following days. For prednisolone treatment by gavage, measurements were shortly interrupted daily between 1200 and 1300 h. Oxygen and carbon dioxide flow rates were measured every 13 min. The respiratory exchange ratio (RER) was calculated using the following equation: RER = VO₂/ VCO₂, in which the VO₂ represents the volume rate of oxygen consumption and the VCO₂ is the volume rate carbon dioxide production.

Measurement of mass isotopomer distribution by GC-MS

The fractional contributions of stable isotopically labeled carbohydrates from blood and urine samples collected during the HIEC and HGC and during the blood glucose kinetics study were extracted, derivatized, and measured by GC-MS as previously described by van Dijk et al. (25).

Metabolite analysis

Basal values of blood glucose concentrations were measured in conscious mice in blood drops collected by tail tip bleeding with a Lifescan EuroFlash glucose meter. Plasma insulin, corticosterone, and fibroblast growth factor-21 (FGF21) concentrations were measured using commercially available ELISA kits (Mercodia ultrasensitive mouse insulin ELISA; Orange Medical, Tilburg, The Netherlands; Diagnostic System Laboratories Benelux, Assendelft, The Netherlands; and Millipore, Amsterdam, The Netherlands, respectively). The homeostatic assessment model of insulin resistance (HOME-IR) was calculated adjusted for basal mouse values taken from a chow-fed vehicle-treated group as previously described (18). Plasma nonesterified fatty acid (NEFA) and 3-hydroxybutyrate concentrations were determined with commercially available kits (Diassys, Holzheim, Germany, and Wako Chemicals, Neuss, Germany, respectively). Hepatic lipids were extracted from the liver homogenates according to Bligh and Dyer (28). Hepatic and plasma triglycerides were measured using a kit from Roche Diagnostics (Mannheim, Germany), and free and total cholesterol levels were measured with a kit from Wako Chemicals. Hepatic glycogen concentrations were determined as described (29). Plasma diabetes biomarkers were measured by a commercially available multiplex (Bio-Plex Pro Mouse Diabetes; Bio-Rad, Veenendaal, The Netherlands) measuring insulin, leptin, glucagon, ghrelin, glucagon like peptide-1 (GLP-1), gastric inhibitory peptide (GIP), resistin, and plasminogen activator inhibitor-1 (PAI-1).

Gene expression analysis

RNA was isolated from liver and white adipose tissue, and gene expression was measured as previously described (18). The sequence of the primers and probes that were previously used can be found on http://www.labpediatricsrug.nl and are deposited at RTR PrimerDB (http://www.rtrprimerdb.org). The sequences of the primers and probes used for leptin are (accession no. NM_008493.3) AAG ACC ATT GTC ACC AGG ATC AA (forward primer), GGT CCA TCT TGG ACA AAC TCA GA (reverse primer), and CAC ACA CGT AGT CCG TAT CCG CC (probe).

Statistics

All data are represented as mean ± SEM. Statistical analysis for two independent groups was performed using Mann-Whitney U test with SPSS for Windows version 16 software (SPSS, Chicago, IL). For multiple comparisons, statistical analyses were performed by Kruskal-Wallis test, followed by a Mann-Whitney U test. For paired measurements, statistical analysis was performed using a Wilcoxon signed-rank test. Significance was considered at a level of P < 0.05 and adjusted for multiple comparisons.

Results

Chronic prednisolone treatment aggravates the high-fat diet-induced increase in HOMA-IR

To assess whether chronic prednisolone treatment aggravates high-fat diet-induced insulin resistance, we first
examined the effect of prednisolone treatment on body parameters and metabolite concentrations (Table 1). Effective prednisolone treatment was confirmed by a severe reduction of thymus weight and a reduction of plasma corticosterone concentration by almost 90% due to suppression of the hypothalamic-pituitary-adrenal axis. Prednisolone treatment did not further affect the increase in body weight in mice fed a high-fat diet for 6 wk.

After 9 h of fasting, blood glucose levels were higher upon prednisolone treatment in mice fed a high-fat diet, whereas fasting insulin levels were slightly increased as well, resulting in a 2-fold higher HOMA-IR (Table 1). The high-fat diet itself also increased the HOMA-IR to 3.6 ± 1.3 compared with mice on a chow diet, which had a HOMA-IR of 1.0 ± 0.1 (P = 0.003) (30). Prednisolone treatment in mice fed a high-fat diet increased plasma cholesterol and triglyceride levels, whereas plasma NEFA concentration dropped. Remarkably, prednisolone treatment resulted in a 10-fold decrease in plasma 3-hydroxybutyrate concentrations and almost doubled plasma FGFR2 levels in the fasted state (Table 1) but did not influence hepatic cholesterol and triglyceride levels.

To evaluate whether the prednisolone-induced increase in HOMA-IR influenced fasting glucose production and clearance, we analyzed fasting blood glucose kinetics in the whole body from the washout of ip-injected D-[6,6-2H2]glucose from the circulation. To follow the effects of both the high-fat diet and prednisolone treatment in time, mice were subjected to this test three times: 1) at the start of the experiment (wk 0) before start of the diet, 2) after 5 wk of diet before start of the prednisolone treatment, and 3) after 6 wk of diet and 1 wk of prednisolone treatment. This experimental setup allows for sequential analysis of glucose metabolism under basal conditions throughout the 6 wk of diet in the same animals. After 5 wk on a high-fat diet, blood glucose levels were significantly elevated in high-fat-fed mice compared with mice on a normal chow diet (Fig. 1A). One week of prednisolone treatment resulted in an additional increase of blood glucose levels compared with mice fed a high-fat diet for 6 wk. Plasma insulin levels followed a similar pattern over time (Fig. 1B), showing that plasma insulin levels were elevated upon feeding a high-fat diet. Yet in mice fed a high-fat diet combined with prednisolone treatment, plasma insulin levels were even more strongly induced.

The high-fat diet induced a distinct reduction of metabolic clearance rate (MCR) of glucose after 35 and 42 d compared with a Chow diet at the start of the experiment (Fig. 1C). Treatment with prednisolone had no significant additional effect on MCR. Neither the high-fat diet nor prednisolone treatment changed the HGP (Fig. 1D). Nevertheless, note that plasma insulin levels were extremely high in prednisolone-treated mice fed a high-fat diet. Therefore, when HGP and MCR were related to the pre-existing insulin levels, mice treated with prednisolone upon a high-fat diet appeared insulin resistant, which was in line with the increased HOMA-IR.

### Table 1. Treatment parameters of 9-h-fasted mice fed a high-fat diet for 6 wk and treated with either vehicle (control) or prednisolone (10 mg/kg · d) during the last 7 d

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Prednisolone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 0</td>
<td>25.2 ± 0.57</td>
<td>25.4 ± 0.42</td>
</tr>
<tr>
<td>d 42</td>
<td>29.1 ± 1.2</td>
<td>29.8 ± 0.5</td>
</tr>
<tr>
<td>Thymus weight (mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25.1 ± 2.8</td>
<td>4.1 ± 0.23*</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>7.6 ± 0.3</td>
<td>11.1 ± 0.6*</td>
</tr>
<tr>
<td>Insulin (mU/liter)</td>
<td>7.4 ± 2.6</td>
<td>10.3 ± 1.7</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.6 ± 1.3</td>
<td>7.3 ± 1.3*</td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>5.9 ± 0.1</td>
<td>7.9 ± 0.4*</td>
</tr>
<tr>
<td>Free cholesterol (mM)</td>
<td>1.5 ± 0.08</td>
<td>1.9 ± 0.11*</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.3 ± 0.07</td>
<td>1.9 ± 0.15*</td>
</tr>
<tr>
<td>NEFA (mM)</td>
<td>0.58 ± 0.03</td>
<td>0.44 ± 0.02*</td>
</tr>
<tr>
<td>Corticosterone (μg/liter)</td>
<td>820 ± 37</td>
<td>96 ± 17*</td>
</tr>
<tr>
<td>β-Hydroxybutyrate (mM)</td>
<td>1.07 ± 0.27</td>
<td>0.11 ± 0.01*</td>
</tr>
<tr>
<td>FGFR2 fasted (μg/liter)</td>
<td>1.41 ± 0.30</td>
<td>2.77 ± 0.98*</td>
</tr>
<tr>
<td>Liver (μmol/g liver)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>11.8 ± 0.76</td>
<td>13.6 ± 0.53</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>43.7 ± 6.39</td>
<td>57.8 ± 4.99</td>
</tr>
<tr>
<td>Glycogen</td>
<td>111 ± 29</td>
<td>154 ± 31</td>
</tr>
</tbody>
</table>

Data are represented as means ± SEM; n = 8. *P < 0.05. The HOMA-IR was calculated adjusted for basal mouse values taken from a chow-fed, vehicle-treated group.
Prednisolone does not induce insulin resistance according to the HIEC and HGC

To study whether increased blood glucose and plasma insulin concentration in high-fat-fed mice treated with prednisolone was actually caused by insulin resistance, we combined the HIEC with the MIDA technique to measure hepatic glucose metabolism during hyperinsulinemia. Again, prednisolone treatment led to increased fasting glucose levels (6.1 ± 0.4 vs. 8.8 ± 0.6, vehicle vs. prednisolone, P = 0.004), and glucose levels during the basal period of the test tended to be higher (P = 0.055) (Fig. 2A). During the basal period, glucose infusion rate (GIR) was kept at a steady, very low level in both groups because it represents the infusion of the stable isotopes solution, as is evident from Fig. 2B. During the hyperinsulinemic period, the GIR was adjusted to keep a euglycemic state at a blood glucose level of 7 mM in both groups. Surprisingly, prednisolone treatment on top of a high-fat diet did not influence the magnitude of the GIR (Fig. 2B).

Even though blood glucose levels in the basal state tended to be higher in prednisolone-treated mice compared with controls, both the MCR (Fig. 2C) and HGP (Fig. 2D) were similar during this period. Upon hyperinsulinemia, the well-documented stimulatory effect of insulin on the uptake of glucose in the peripheral tissues and inhibitory effect on the hepatic production of glucose were observed in both groups. Prednisolone treatment did not further alter the MCR but, strikingly, resulted in a more pronounced suppression of the HGP by insulin.

By means of MIDA, the relevant hepatic fluxes constituting hepatic glucose metabolism were measured, and these are presented in Table 2. Prednisolone treatment reduced both the glycogen synthase (GS) flux and the gluconeogenic (GNG) flux in the basal period. The hyperinsulinemia reduced the glucose-6-phosphatase (G6Pase), the glycogen phosphorylase (GP), and GNG flux in both groups. Strikingly, even though GNG has repeatedly been reported to be up regulated by glucocorticoids (31), it was slightly reduced upon prednisolone treatment in both the basal and hyperinsulinemic state in mice on a high-fat diet. In addition, prednisolone treatment led to a reduced GS and GP flux in the hyperinsulinemic period, resulting in a similar glycogen balance compared with the vehicle-treated group in the hyperinsulinemic period compared with the basal period. From these fluxes, the glucose and glycogen balances were calculated (Fig. 2, E and F). The glucose balance was reduced in both groups during the hyperinsulinemic period, mainly due to a reduction in G6Pase flux. This was even more pronounced in the prednisolone-treated group. The initially negative glycogen balance, which was more negative in prednisolone-treated mice, turned positive upon hyperinsulinemia, even more pronounced in prednisolone-treated mice. The differences in glycojen balance upon hyperinsulinemia were mainly due to reductions in the GP flux, whereas the GS flux was not affected by hyperinsulinemia but was reduced by prednisolone treatment in both periods.

So far, these data suggest that prednisolone treatment of mice receiving a high-fat diet did not decrease insulin sensitivity as measured with the gold standard HIEC, although blood glucose and plasma insulin levels as well as hepatic glucose metabolism were clearly affected. To evaluate regulation of glucose metabolism during stimulation of endogenous insulin production at elevated glucose levels, HGC combined with hepatic glucose metabolism analysis was performed. To this purpose, mice were fasted for 4 h, after which the groups did not show a difference in fasted blood glucose levels (8.6 ± 0.3 vs. 8.2 ± 0.4 mM, means ± SEM, vehicle vs. prednisolone, not significant) and in blood glucose levels in the basal period (Fig. 3A). To induce hyperglycemia with blood glucose levels up to 17 mM, very similar GIR were necessary in both groups (Fig. 3B), indicating no differences in insulin response because insulin levels during the HGC were similar in both groups.
TABLE 2. Hepatic glucose fluxes during the basal and hyperinsulinemic period

<table>
<thead>
<tr>
<th>Fluxes</th>
<th>Basal period</th>
<th>Hyperinsulinemic period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Prednisolone</td>
</tr>
<tr>
<td>G6Pase</td>
<td>170.4 ± 5.9</td>
<td>190.3 ± 8.3</td>
</tr>
<tr>
<td>GK</td>
<td>58.5 ± 4.2</td>
<td>68.6 ± 8.1</td>
</tr>
<tr>
<td>GS</td>
<td>59.1 ± 5.8</td>
<td>39.4 ± 1.5a</td>
</tr>
<tr>
<td>GP</td>
<td>65.9 ± 4.5</td>
<td>65.1 ± 5.5</td>
</tr>
<tr>
<td>GNG</td>
<td>105.1 ± 1.9</td>
<td>95.9 ± 2.9a</td>
</tr>
</tbody>
</table>

The hepatic carbohydrates fluxes through G6Pase, GK, GS, GP, and GNG are shown in micromoles per kilogram per minute. Values represent means ± SEM; n = 7–8.

a P < 0.05, prednisolone vs. vehicle.

b P < 0.05, hyperinsulinemic vs. basal period within treatment.

[41.8 (11.7–73.1) vs. 25.9 (11.6–74.0) mU/liter after the hyperglycemic period, median and ranges, vehicle vs. prednisolone, not significant].

In the basal period, MCR and HGP were similar in both groups, as expected from the former experiment. Upon hyperglycemia, MCR was increased in both groups, whereas HGP showed a slight decrease in vehicle-treated mice (Fig. 3, C and D). Prednisolone treatment had no effect on either MCR or HGP. Nevertheless, analysis of hepatic glucose fluxes revealed that the GS flux was clearly reduced in the hyperglycemic period by prednisolone treatment (Table 3). This resulted in a minor effect on glycogen balance (Fig. 3F). The glucose balance was reduced in the vehicle group during the hyperglycemic period (Fig. 3E), which was mainly due to an increase in the glucokinase (GK) flux, which remained unchanged in the prednisolone-treated group.

We checked for hepatic mRNA expression levels of genes crucially involved in glucose metabolism (Fig. 4): phosphoenolpyruvate carboxykinase 1 (Pck1) and G6Pase (G6pc) were reduced by a high-fat diet compared with chow and combined with prednisolone even more so. GK (Gck) and pyruvate kinase (Pklr) mRNA levels were increased by the high-fat diet or prednisolone treatment, respectively. Glucocorticoid target genes were checked to confirm prednisolone activation. Surprisingly, the classical glucocorticoid target gene tyrosine aminotransferase (Tat) was reduced by a high-fat diet and unaltered by prednisolone, whereas Pck1, another classic target gene, was even reduced by prednisolone treatment.

**Prednisolone alterations on blood glucose metabolism are insulin independent**

The apparent insulin resistance as indicated by an increased HOMA-IR and blood glucose kinetics under prevailing insulin concentrations was not revealed when insulin sensitivity was more thoroughly studied by the HIEC and the HGC. This suggested that prednisolone treatment in mice fed a high-fat diet did not induce a classical insulin resistance measured according to generally accepted methods. These findings could imply that other metabolic factors might be involved. As shown in Table 1, plasma FGF21 levels were increased upon prednisolone treatment, which was confirmed by hepatic gene expression levels of Fgf21 (Fig. 5A). The main regulator of FGF21 expression, peroxisome proliferator-activated receptor α (Ppara) (18), showed no increase in gene expression levels.
and target genes of Ppara, such as Acox1 and Cpt1a, were not elevated by prednisolone.

Finally, we analyzed multiple hormones and hormone-like factors in plasma from fed and 9-h-fasted mice. Table 4 shows that prednisolone induced plasma leptin levels, both in the fed and fasted state, in addition to inducing plasma insulin levels. Other analyzed hormones were not affected by prednisolone treatment in the fasted state, but PAI-1 was increased upon prednisolone treatment in the fed state only. Fasting itself reduced plasma leptin, GLP-1, and GIP. Increased plasma leptin levels were confirmed by leptin gene expression levels in white adipose tissue (Fig. 5B). Leptin plays a major role in energy expenditure and as a satiety factor; therefore, energy expenditure in mice fed a high-fat diet was measured in indirect calorimetry cages. Figure 5C shows a slight increase in RER at night in prednisolone-treated mice, indicating that at night, glucose utilization is increased in mice fed a high-fat diet treated with prednisolone compared with vehicle-treated mice. Food intake, however, was slightly increased upon prednisolone treatment (Fig. 5D).

Discussion

In an earlier study (18), we showed that, under chow-fed conditions, sustained treatment of C57BL/6J mice with...
prednisolone reduced insulin sensitivity only in a very subtle manner under fasting conditions. In this subsequent study, we show that in mice challenged with a high-fat diet, prednisolone treatment did not aggravate insulin resistance induced by a high-fat diet as measured by HIEC, even though these mice appeared insulin resistant because HOMA-IR was increased and whole-body glucose kinetics at basal prevailing insulin concentrations were perturbed. This study clearly shows a disengagement of the HOMA-IR with outcome of the HIEC and HGC and also documents that prednisolone-induced alterations of glucose metabolism in these mice are not directly linked to insulin sensitivity.

Insulin resistance is a well-known side effect of glucocorticoid treatment in humans (8, 9, 32). In addition, it has been shown repeatedly that dexamethasone treatment also induces insulin resistance in rats (11, 13, 33, 34). In mice, however, glucocorticoid-induced insulin resistance is not as evident. Most studies on glucocorticoid-induced insulin resistance in mice involve specific transgenic mouse strains (15, 35) usually in combination with long-term, or high-dose, dexamethasone administration (36, 37). Because prednisolone is routinely used for chronic treatment in conventional C57BL/6J mice we subjected them to different methods available to assess insulin resistance, including the HOMA-IR, HIEC, HGC, and basal blood glucose kinetics, and analyzed hepatic glucose metabolism by MIDA.

Like in chow-fed mice (18), we confirmed that chronic, 7-d prednisolone treatment increased the HOMA-IR in high-fat-fed mice, whereas, as expected, the high-fat diet by itself already caused an increased HOMA-IR in comparison with chow-fed mice. Note that for the calculations of HOMA-IR, the values were adjusted for basal values of glucose and insulin concentrations in mice. In addition, when the MCR and HGP calculated from basal whole-body blood glucose kinetics were related to prevailing insulin levels, prednisolone-treated mice appeared to have reduced insulin sensitivity. Yet, similar to our earlier study in chow-fed animals, when insulin sensitivity was evaluated by the HIEC, the gold standard to evaluate insulin action on glucose metabolism, prednisolone treatment was found not to alter insulin sensitivity because GIR values were similar with and without prednisolone treatment. This clearly shows that, although HOMA-IR and HIEC were shown to be well correlated in humans (40), there is a disengagement between the HOMA-IR with the HIEC in mice, which has previously been reported for rodents (39, 41). Nevertheless, assessment of hepatic carbohydrate metabolic fluxes by stable isotope techniques under basal and hyperinsulinemic-euglycemic as well as hyperglycemic conditions revealed that prednisolone treatment did alter hepatic glucose metabolism in high-fat-fed mice, mainly by reducing the glycogen synthase flux. These effects of prednisolone on glucose metabolism are not mediated by insulin, because infusion of insulin during HIEC did not affect the GS flux but, in contrast, reduced the GP flux.

In our former study (18), we showed that nutritional status has to be taken into account when evaluating prednisolone-induced insulin resistance. To accurately assess effects of prednisolone on glucose metabolism in mice fed the high-fat diet, we studied hepatic glucose metabolism under different nutritional conditions, i.e., after 4 and 9 h of fasting and under hyperglycemic and hyperinsulinemic euglycemic conditions. First, the 9-h-fasted state was assessed by measuring blood glucose kinetics by modeling

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**TABLE 4.** Plasma concentrations of diabetes biomarkers

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Prednisolone</th>
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</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>5.2 ± 1.8</td>
<td>10.2 ± 3.8</td>
</tr>
<tr>
<td>Glucagon</td>
<td>0.70 ± 0.14</td>
<td>0.79 ± 0.22</td>
</tr>
<tr>
<td>Leptin</td>
<td>22.2 ± 4.3</td>
<td>44.5 ± 7.9</td>
</tr>
<tr>
<td>Resistin</td>
<td>261.7 ± 72.3</td>
<td>229.6 ± 64.4</td>
</tr>
<tr>
<td>GLP-1</td>
<td>0.19 ± 0.05</td>
<td>0.17 ± 0.08</td>
</tr>
<tr>
<td>GIP</td>
<td>1.16 ± 0.35</td>
<td>1.47 ± 0.54</td>
</tr>
<tr>
<td>PAI-1</td>
<td>0.82 ± 0.07</td>
<td>1.45 ± 0.28</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>3.58 ± 1.25</td>
<td>2.41 ± 1.39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Prednisolone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>ND</td>
<td>0.38 ± 0.06</td>
</tr>
<tr>
<td>Glucagon</td>
<td>7.72 ± 2.96</td>
<td>7.27 ± 3.04</td>
</tr>
<tr>
<td>Leptin</td>
<td>217.1 ± 43.1</td>
<td>227.7 ± 57.4</td>
</tr>
<tr>
<td>Resistin</td>
<td>0.11 ± 0.02</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>GLP-1</td>
<td>0.40 ± 0.07</td>
<td>0.37 ± 0.10</td>
</tr>
<tr>
<td>GIP</td>
<td>1.08 ± 0.32</td>
<td>1.04 ± 0.34</td>
</tr>
<tr>
<td>PAI-1</td>
<td>6.95 ± 2.37</td>
<td>4.78 ± 2.00</td>
</tr>
</tbody>
</table>

Plasma concentrations are shown as micrograms per milliliter. Fed samples were taken at 2300 h and fasted samples at 0800 h after a 9-h fast. Values represent means ± SD; n = 6. ND, Not detectable.

* a P < 0.05, prednisolone vs. vehicle.

* b P < 0.05, fed vs. fasted.
the washout of ip-injected \( \delta^{-6,6-2H_2} \)glucose, because this method allows repeated measurements over time in the same animals. This is not possible with the HIEC and HGC, because these are complex and laborious experiments that require invasive surgery. However, to quantitatively assess hepatic glucose metabolism and hepatic and peripheral insulin resistance, both the HIEC and HGC were combined with a method to study hepatic glucose metabolism with stable isotopically labeled glucose by MIDA (25–27). By adapting these state-of-the-art methods, both hepatic carbohydrate fluxes and insulin sensitivity could be measured at the same time. During the HIEC, supraphysiological insulin levels were introduced, which can be regarded as a postprandial condition. Importantly, during the HIEC, the pancreas is blocked by somatostatin, thereby overruling part of the body’s natural reaction. To aim for a more physiological approach with a normal pancreatic response at high glucose levels, the HGC was used. The basal periods of the HIEC and the HGC both symbolize a different nutritional status; for the HIEC, mice were fasted for 9 h, whereas for the HGC, mice were fasted for only 4 h. During the basal periods of both experiments, the sum fluxes from both hepatic glucose sources, i.e. glycogen phosphorylase and gluconeogenesis, was identical. However, there is a switch from the glycogen phosphorylase to gluconeogenesis with prolonged fasting. This shows that upon fasting, first glycogen breakdown takes place after which gluconeogenesis takes over. Note that the magnitude of HGP and MCR in the basal period of both clamp experiments is in the same order of magnitude as the HGP and MCR from the fasting blood glucose kinetics, confirming the relevance of this experimental procedure. During the HIEC, GIR were equal between the groups; however, the HGP was slightly reduced during the hyperinsulinemic period in prednisolone-treated mice. Because this was at similar infusion rates of insulin, the reduced HGP may be explained as increased hepatic insulin sensitivity. Results in our previous study suggested that the hyperinsulinemia could mask the effects of subtle changes induced by prednisolone on insulin sensitivity. In the current study, these subtle changes should be revealed during the HGC in which the HGP and MCR are regulated by endogenous prevailing insulin concentrations. However, also during the HGC, the prednisolone-treated animals appeared to be equally insulin sensitive.

Relating hepatic gene expression levels of the genes encoding enzymes that catalyze the reactions that constitute the hepatic carbohydrate fluxes to the quantitatively measured hepatic glucose fluxes is complex; fluxes were measured over a period of time under a specific nutritional status, whereas samples for gene expression level measurements were taken at fixed time points after fasting and treatment. In addition, regulation of metabolic fluxes largely depends on the substrate availability and enzyme activity. The observed effects of the chronic prednisolone treatment in the current study on mRNA levels of glucocorticoid receptor target genes are partly contrasting reported effects. Tat (42) and Pck1 (43, 44) have been shown to be classical glucocorticoid receptor target genes, being up-regulated when glucocorticoids are present, but prolonged prednisolone treatment, like in our study, resulted in decreased hepatic mRNA levels of both genes, as previously reported (18). Up-regulatory effects of glucocorticoids on Pck1 have been shown to be fast and transient (45). Thus, the reduced Pck1 expression likely reflects the reduced plasma glucocorticoid concentration in prednisolone-treated mice 18 h after the last dose. In addition, a study by Magnuson et al. showed actually that major changes in gene expression of Pck1 do not alter GNG flux per se (46).

As previously shown in mice receiving a chow diet, also on a high-fat diet, plasma FGF21 levels were elevated by prednisolone (18). Together with the low plasma 3-hydroxybutyrate levels, high FGF21 levels imply a perturbed feeding-to-fasting transition (47, 48) also in mice fed a high-fat diet. Possibly, prednisolone-treated mice might be FG21 resistant, like in obesity (49). In addition, FGF21 has been shown to reduce G6pc and glycogenolysis through Pgc1α (50); prednisolone-treated mice in this study had a reduction in hepatic G6pc gene expression levels, which could be mediated via FGF21-Pgc1α.

During fasting, leptin is reduced, thereby reducing satiety and promoting food intake (51). In mice on a high-fat diet, prednisolone treatment increased plasma leptin levels both in the fed and the fasted state but did not reduce food intake. In addition, the body weight of prednisolone-treated mice did not increase; therefore, the augmented levels of leptin are not due to increased adipose tissue content (52). Leptin and glucocorticoids are counterregulatory and control satiety and appetite mainly via the hypothalamus (53). Moreover, together with insulin, leptin and glucocorticoids are important factors in an expanding hormonal network regulating glucose metabolism, which in turn is tightly connected to energy homeostasis as well as regulation of muscle and adipose tissue metabolism. It was thought that hepatic leptin signaling did not affect hepatic glucose metabolism (54, 55), yet a recent paper showed that the hepatic leptin receptor is involved in diet- and age-related insulin resistance (56), thereby opening a direct role for leptin on hepatic glucose metabolism. These data demonstrate that prednisolone, which caused a subtle phenotype on glucose metabolism in C57BL/6J mice, closely interacts with mem-
bers of a complex hormonal network influencing glucose metabolism other than insulin.

Although glucocorticoids have been shown to induce insulin resistance in humans and rats measured by HIEC, prednisolone treatment in C57BL/6J mice fed a high-fat diet did not lead to such a clear insulin-resistance phenotype. Nevertheless, fasting glucose and insulin levels, fasting blood glucose kinetics, and hepatic glucose fluxes during both hyperinsulinaemic and hyperglycaemic states showed clear signs of an adapted glucose metabolism after prednisolone treatment. In addition, this study highlights discrepancies between the effects of prednisolone in mice and men, in the sense that clear-cut insulin resistance is not induced in the first, not even when challenged with a high-fat diet. Yet also in humans, effects of prednisolone on insulin sensitivity are highly variable (57). Interestingly, this study provides valuable new information regarding effects of prednisolone on hormonal systems, which may be of relevance for the human situation. We conclude that although prednisolone treatment did not aggravate high-fat diet-induced insulin resistance in mice, it does alter hepatic glucose fluxes independent of insulin action. This is presumably due to adaptations in the hormonal networks regulating glucose control, involving leptin and FGF21.

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Address all correspondence and requests for reprints to: Department of Pediatrics, CMCIV Room Y2.175, University Medical Center Groningen, P.O. Box 30.001, 9700 RB, Groningen, The Netherlands. E-mail: a.k.groen@umcg.nl.

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Disclosure Summary: The authors have nothing to disclose.

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