Glucocorticoid receptor haploinsufficiency causes hypertension and attenuates hypothalamic-pituitary-adrenal axis and blood pressure adaptions to high-fat diet

Z. Michailidou,* R. N. Carter,* E. Marshall,* H. G. Sutherland,† D. G. Brownstein,* E. Owen,* K. Cockett,* V. Kelly,* L. Ramage,* E. A. S. Al-Dujaili,‡ M. Ross,* I. Maraki,* K. Newton,† M. C. Holmes,* J. R. Seckl,* N. M. Morton,* C. J. Kenyon,* and K. E. Chapman*†

*Centre for Cardiovascular Sciences, Queen’s Medical Research Institute, University of Edinburgh, Edinburgh, UK; †Medical Research Council Human Genetics Unit, Edinburgh, UK; and ‡Queen Margaret University, Musselburgh, East Lothian, UK

ABSTRACT Glucocorticoid hormones are critical to respond and adapt to stress. Genetic variations in the glucocorticoid receptor (GR) gene alter hypothalamic-pituitary-adrenal (HPA) axis activity and associate with hypertension and susceptibility to metabolic disease. Here we test the hypothesis that reduced GR density alters hypertension and susceptibility to metabolic disease. Pituitary-adrenal (HPA) axis activity and associate with glucocorticoid receptor (GR) gene alter hypothalamic-adapt and adapt to stress. Genetic variations in the

*Correspondence: Endocrinology Unit, Centre for Cardiovascular Sciences, The Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK. E-mail: karen.chapman@ed.ac.uk

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associated with hyper- or hyposensitivity to exogenous glucocorticoid (dexamethasone) suppression of the hypothalamic-pituitary-adrenal (HPA) axis and a range of metabolic and cardiovascular parameters. Thus, polymorphisms associated with glucocorticoid hypersensitivity (Bcl, N363S) are typically (dependent on age, gender, and ethnic background) associated with hypertension, obesity, decreased lean mass, insulin resistance, and increased risk of cardiovascular disease despite reduced plasma cortisol levels, whereas the ER22/23EK polymorphism, associated with glucocorticoid hyposensitivity, is associated with beneficial metabolic outcomes (reviewed in ref. 14).

Several mouse lines with altered GR expression or function have been generated to investigate the role of GR in HPA axis regulation and its role in mood regulation and in the immune system (reviewed in refs. 15–17). Fewer studies have investigated the effects of altered GR density on cardiovascular and metabolic outcomes. Reduced GR density through antisense GR transgene expression (central nervous system targeted, although widely expressed) lowered energy intake but also caused obesity (18, 19). However, neuron-specific deletion of GR caused a small-lean phenotype postweaning and increased energy expenditure, due to the peripheral effects of high glucocorticoid levels coupled with normal levels of peripheral GR (20). Liver-specific GR gene deletion led to hypoglycemia, but only after prolonged starvation, and ameliorated hyperglycemia in streptozotocin-induced diabetes (21). Recently, transgenic mice have been described with cardiomyocyte-specific GR overexpression. These mice display conduction defects, reduced heart rate, atrioventricular block, altered calcium homeostasis, and ion channel remodeling in isolated cardiomyocytes (22). Although these studies have provided important information on the tissue-specific functions of GR, the cardiovascular and metabolic consequences of globally altered GR density, and hence glucocorticoid sensitivity, have not been described, although this is the clinically relevant situation.

Here we have generated a novel line of mice with reduced GR density, heterozygous for a null mutation of the GR gene ($GR^{geo/+}$), to investigate whether global reduction in GR density alters cardiovascular (blood pressure), fat distribution, and metabolic (glucose and lipid homeostasis) parameters. A high-fat (HF) diet was introduced to determine whether GR haplinsufficiency altered the adaptive hormonal and metabolic changes that accompany dietary-induced obesity.

**MATERIALS AND METHODS**

**Generation of $GR^{geo/+}$ mice**

A mouse ES cell line (ESKN92) generated by gene-trap mutagenesis in which a β-galactosidase-neomycin phosphotransferase (βgeo) reporter cassette integrated within the GR gene, generating a translational fusion between GR and βgeo, has been described previously (23). 5'-Rapid amplification of cDNA ends (RACE) confirmed integration of the β-galactosidase-neomycin phosphotransferase (βgeo) reporter cassette between exons 3 and 4 of the GR gene. Fluorescence in situ hybridization (FISH) confirmed a single integration site on chromosome 18 at the GR locus. Chimeric mice were generated by injection of ESKN92 cells into C57BL/6J blastocysts. Chimeras were mated to C57BL/6J female mice to achieve germ line transmission and further backcrossed to generate a congenic line (full name, Nv3:RMs6SkgeoMc/McJ; here abbreviated to $GR^{geo}$). Heterozygotes were identified by polymerase chain reaction (PCR) for the presence of lacZ (in the βgeo cassette) using the following primers: forward, 5'-GGTGG-GCAAGCTTGAATGGCG-3'; and reverse, 5'-GCCGTGACTGC-CAACGCGAAG-3'. All the experiments described were performed on adult male (5–6 months) $GR^{geo/+}$ and $GR^{geo/-}$ litters, backcrossed for five generations (F5) to C57BL/6J. Experimental groups were 8 to 9 per group, unless otherwise stated. To generate homozygous $GR^{geo/geo}$ mice, F1 $GR^{geo/+}$ mice were intercrossed.

**Animal maintenance and diets**

All animal experimentation was conducted in strict accord with the accepted standards of humane animal care under the auspices of the Animal (Scientific Procedures) Act UK 1986 after prior approval by the local ethical committee. Mice were housed in standard cages under controlled lighting (12:12-h light-dark cycle, lights on at 7 AM) and were fed standard chow from weaning unless specified otherwise. For measurement of unstressed plasma corticosterone ACTH and glucose in tail nick blood samples, animals were acclimatized to single housing and tail nicks were performed within 1 min of disturbing the cage. For diet-induced obesity experiments, mice were weaned onto either HF/low carbohydrate diet (58% kcal as fat, D12331; Research Diets, New Brunswick, NJ, USA) or low-fat (LF)/high-carbohydrate diet (11% kcal as fat, D12328; Research Diets) and remained on the diet for 22 wk with ad libitum access to water and diet. Body weight and food intake were monitored weekly and for 3 wk, respectively.

**Tissue collection, metabolic parameters, and liver triglyceride levels**

Experimental mice were killed by decapitation between 8 and 10 AM. Trunk blood samples were collected into EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) and centrifuged (6000 g, 10 min), and plasma was stored at −80°C before assay. Tissues were rapidly frozen in dry ice for RNA or Western blot analysis or fixed in formalin (left adrenal, kidney) for histology. Evening plasma corticosterone levels were determined from blood sampled (by tail nick) at 7 PM. Plasma corticosterone was measured by an in-house radioimmunoassay as described previously (24). Plasma ACTH levels were measured by ELISA (Biomera, Newport Beach, CA, USA). For the glucose tolerance test (GTT), animals were deprived of food for 6 h, 2 mg/g body weight of 25% glucose was injected intraperitoneally, and tail nick blood sampling was performed at time 0 (before injection) and 15, 30, 60, and 120 min after injection. Plasma glucose was measured using a glucose monitoring system (One Touch Ultra, Lifespan, Johnson & Johnson, Langhorne, PA, USA), insulin by ELISA (Crystalchem, Downers Grove, IL, USA), nonesterified fatty acids (NEFAs) with a NEFA C kit (Wako Chemicals GmbH, Nuess, Germany), and triglycerides with an L-type triglyceride kit (Wako Chemicals GmbH). Insulin, NEFAs, and triglycerides were measured after 24 h of food deprivation. Hepatic triglycerides were extracted after homogenization of 100 mg liver in isopropanol (10 vol) and then incubation at 37°C for 45 min. After centrifugation (3000 g, 10 min), 10 µl supernatant was incubated at 37°C for 5 min with 1 ml Thermotrace triglyceride
reagent (Alpha Laboratories, Eastleigh, UK) and absorbance at 500 nm was measured. Plasma renin activity and angiotensinogen concentration were measured by radioimmunoassay as described previously (25). Plasma aldosterone concentration was measured by in-house ELISA as described previously (26, 27). Formalin-fixed tissues were processed for histopathology, sectioned (4 μm), and stained with hematoxylin and eosin for histopathological examination.

Blood pressure measurements

Systolic blood pressure was measured on 2 separate days in conscious mice by tail cuff plethysmography (Harvard Apparatus, Edenbridge, UK) as described previously (28). Before measurements were recorded, all mice underwent three periods of training to acclimatize them to the procedure. Mice were kept at 37°C for 10 min before measurements were initiated. Mean systolic blood pressure was calculated from the mean of 12 measurements per mouse.

RNA extraction and real-time PCR

Total RNA was extracted from frozen tissues as described previously (25). One microgram of RNA was pretreated with DNase I (Invitrogen, Paisley, UK) and then reverse transcribed into cDNA using oligo(dT) primer and Superscript III first strand cDNA synthesis kit (Invitrogen). Real-time PCR was carried out on cDNA using a LightCycler 480 (Roche Diagnostics, Burgess Hill, UK) with a Real-time PCR was carried out on cDNA using a LightCycler 480 (Roche Diagnostics, Burgess Hill, UK) with a Real-time Cycler 480 (Roche Diagnostics, Burgess Hill, UK) with a Real-time

Statistical analysis

The effects of genotype and diet were assessed by two-way ANOVA followed by post hoc Tukey’s tests for group differences. Significance was set at P < 0.05. To evaluate significant differences in GR mRNA levels between GR WT and GR GRβgeo mice, a Student’s t test was used. For GTT comparisons, the area under the curve was calculated for each animal and then group means compared with 2-way ANOVA. For transfection assays, data were analyzed by ANOVA, followed by post hoc tests. Values are means ± se.

RESULTS

GRβgeo allele is a null allele that decreases functional GR levels in heterozygous mice

Generation of GRβgeo/+ mice

GRβgeo/+ mutant mice were generated from an ES cell line (ESKN92) in which a βgeo reporter cassette had

Western blotting

Fifty milligrams of epididymal fat tissue was homogenized in 600 μl protein extraction buffer (Invitrogen). Proteins (25 μg homogenate) were resolved on 4–12% Bis-Tris Novex precast Gels (Invitrogen), transferred to a nitrocellulose membrane, and then incubated with a GR-specific antibody (M-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized by chemiluminesence (ECL kit; Amersham Biosciences, Little Chalfont, UK). An anti-β-tubulin monoclonal antibody (Sigma Aldrich, St. Louis, MO, USA) or membrane staining with Ponceau red were used to verify equal protein loading between samples.

X-gal staining

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining to detect β-galactosidase activity was carried out on frozen coronal brain sections (10 μm). Sections were transferred directly from −80°C into fixative (4% paraformaldehyde, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 5 mM EGTA, and 2 mM MgCl₂) for 15 min at 4°C; washed twice in PBS containing 2 mM MgCl₂, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate; and stained for 6 h in PBS containing 2 mM MgCl₂, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 5 mM potassium ferriy-canide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal.

Transfection assays

Human embryonic kidney (HEK293) cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C, 5% CO₂. For transfection, 2.5 × 10⁵ cells were seeded per well in 6-well plates coated with poly-L-lysine in DMEM supplemented with 10% charcoal-stripped fetal calf serum and transfected the following day using Lipofectamine 2000 (Invitrogen) with 500 ng reporter plasmid, 200 ng expression plasmid and/or empty vector, pcDNA3.1(−) (Invitrogen), and 50 ng pRL-CMV (Promega, Southampton, UK) encoding renilla luciferase, used as internal control. Dexamethasone (1 μM) was added 1 h after transfection, and the cells were harvested 48 h later for luciferase assays. Reporter plasmids were MMTV-LTR-luciferase (30) or rPNMT-998/466 Luc (31). Expression plasmids encoded wild-type (WT) mouse GR or GRβgeo. WT GR was subcloned from pSV2wrec (32). A polymorphism within the DNA binding domain of the pSV2wrec-encoded GR (encoding Val437) (33) was changed to Gly437, to be identical to the C57BL/6J and 129 mouse strain-encoded GR, by site-directed mutagenesis. The GRβgeo cDNA was assembled from the WT GR cDNA, the 5’-RACE product, and the pGT3 vector in the gene trap (23) by subcloning of appropriate DNA fragments. All constructs were verified by DNA sequencing.

In situ mRNA hybridization

Brain and pituitary GR mRNA levels were determined by in situ mRNA hybridization histochemistry, as described previously (29). Briefly, coronal brain sections (10 μm) were hybridized overnight at 55°C to 35S-labeled cRNA probe complementary to exons 5–9 of GR (29, absent from ESKN92-encoded GRβgeo mRNA). Sections were then treated with RNase at 37°C for 1 h and washed at 60–70°C. Hybridized sections were exposed to autoradiographic film for 7 days. For densitometry, autoradiographs were imaged on a lightbox fitted with a cool snap photometscamera and analyzed using MCID software (InterFocus Imaging, Cambridge, UK).

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integrated into the GR gene, generating a transcriptional and translational fusion (23). 5'-RACE carried out on ESKN92 RNA and PCR analysis of genomic DNA demonstrated the site of integration between exons 3 and 4 (data not shown). The resulting fusion protein lacks part of the DNA binding domain and the entire ligand binding domain (Fig. 1A).

The encoded protein is transcriptionally inactive

The ability of the GR-βgeo fusion protein to activate transcription was tested in transfected HEK293 cells, which lack functional endogenous GR. In contrast to WT GR, the GR-βgeo fusion protein had no effect on promoter activity either in the presence or absence of dexamethasone (Fig. 1B, C), demonstrating that it is transcriptionally inactive and that the exon 2–3 encoded portion of GR, present in the fusion protein, has no constitutive activity. Importantly, cotransfection of GR-βgeo with WT GR had no effect on dexamethasone-mediated WT GR transactivation of either MMTV-LTR (Fig. 1B) or the PNMT promoter (Fig. 1C), demonstrating that the fusion protein does not exert a dominant-negative activity. Thus, although the fusion protein is expressed in GR-βgeo/+ mice (e.g., Fig. 2), these properties predict that the GR-βgeo allele is a null allele.

Homozygous GR-βgeo/βgeo mice die postnatally

Previous data have shown that homozygosity for a null allele of GR is lethal (34), whereas ∼10–15% of mice homozygous for a hypomorphic allele survive (11). To test the lethality of the GR-βgeo allele, heterozygous GR-βgeo/+ mice were intercrossed. Of 145 offspring, none were homozygous for the GR-βgeo allele, suggesting that homozygous mice die before adulthood, consistent with previous data showing the lethality of a null allele around birth (11, 34). In contrast, examination of embryos from heterozygous intercrosses showed the presence of homozygous GR-βgeo/βgeo embryos at the expected frequency (26.6%).

Functional GR levels are reduced in GR-βgeo/+ mice

X-gal staining of brain sections from GR-βgeo/+ mice showed expression of GR-βgeo throughout the brain, mirroring normal GR mRNA expression (Fig. 2A, B). Moreover, quantitative PCR (qPCR) measurements of GR mRNA levels in GR-/+ and GR-βgeo/+ mice using primers that span exons 2–3 (present in both normal GR and GR-βgeo alleles) did not differ between genotypes (data not shown). However, in situ mRNA hybridization histochemistry of GR mRNA, using a probe complementary to exons 5–9 of GR (absent from mRNA encoding GR-βgeo; Fig. 1A) showed a halving in the full-length GR mRNA levels in the brain (hippocampus and paraventricular nucleus of the hypothalamus) and pituitary of GR-βgeo/+ mice compared with GR+/+ littermates (Fig. 2B–D; Table 1). Similarly, qPCR to detect exons 5–6 showed that GR-βgeo/+ mice had ∼50% reduced full-length GR mRNA levels in adipose tissue, muscle, liver, and adrenal gland (Table 1). Interestingly, the decrease measured in the adrenal gland was significantly greater than that in adipose tissue and liver (P<0.05; repeated one-way ANOVA), possibly reflecting developmental effects. Western blot analysis showed similar reductions in a 95 kDa GR protein in GR-βgeo/+ mice (Fig. 3). A 191 kDa immunoreactive protein, corresponding to the predicted mass of the GR-βgeo fusion protein, was detected in GR-βgeo/+ but not in GR+/+ mouse tissues (Fig. 3).

GR-βgeo/+ mice show altered HPA adaption to HF diet

Differing HPA axis alterations in response to HF diet in GR-βgeo/+ mice

HF diets stimulate the HPA axis (35) and induce hypertension (36, 37). It is well known that GR manipulations affect the HPA axis (15). To establish that HPA axis activity is increased in GR-βgeo/+ mice and to determine whether reduced GR levels alter the metabolic adaption to an HF diet, GR-βgeo/+ mice were weaned onto a defined HF or LF diet and maintained on the
diets for 22 wk. Although on the LF diet GR\textsuperscript{geo/+} mice had elevated plasma corticosterone levels (basal and peak) compared with GR\textsuperscript{+/+} mice (Fig. 4), consistent with the predicted increase in basal HPA axis activity in GR deficiency (15), they resisted the HF diet-induced increase in basal (morning) plasma corticosterone levels (Fig. 4). Thus, while the HF diet increased basal corticosterone levels in GR\textsuperscript{+/+} mice by \(2\)-fold, it had no significant effect on basal corticosterone levels in GR\textsuperscript{geo/+} mice, resulting in no difference between mice of the two genotypes fed the HF diet (Fig. 4). Peak circulating corticosterone levels remained higher in GR\textsuperscript{geo/+} mice compared with their GR\textsuperscript{+/+} littermates and were unaffected by diet (Fig. 4B). There was no significant difference between genotypes in basal (morning) plasma ACTH levels in either LF-fed (data not shown) or HF-fed mice (GR\textsuperscript{+/+} vs. GR\textsuperscript{geo/+}: 38±8 and 19±7 pg/ml, respectively), consistent with the similar basal plasma corticosterone levels in the latter. Similarly, there were no significant effects of either genotype or diet on pituitary POMC mRNA levels, although there was a trend for the HF diet to increase

### TABLE 1. GR\textsuperscript{geo/+} mice have half of the normal GR mRNA levels in the brain and peripheral tissues

| Tissue            | GR\textsuperscript{+/+} | GR\textsuperscript{geo/+} | Reduction (%) | \(P\) value |
|-------------------|-------------------------|---------------------------|---------------|-------------|
| **Brain**         |                         |                           |               |             |
| Hippocampus       |                         |                           |               |             |
| CA1               | 0.4 ± 0.05              | 0.2 ± 0.03                | 50            | 0.002       |
| CA2               | 0.5 ± 0.06              | 0.2 ± 0.05                | 60            | 0.003       |
| CA3               | 0.14 ± 0.01             | 0.06 ± 0.02               | 57            | 0.002       |
| DG                | 0.4 ± 0.03              | 0.2 ± 0.03                | 50            | 0.002       |
| PVN               | 0.4 ± 0.1               | 0.2 ± 0.05                | 50            | 0.05        |
| Pituitary         | 0.5 ± 0.07              | 0.2 ± 0.04                | 60            | 0.002       |
| **Periphery**     |                         |                           |               |             |
| Adren gland       | 1.7 ± 0.3               | 0.6 ± 0.08                | 65            | 0.002       |
| Liver             | 1.0 ± 0.05              | 0.7 ± 0.06                | 30            | 0.002       |
| Epididymal fat    | 1.3 ± 0.09              | 0.7 ± 0.05                | 46            | 0.0002      |
| Inguinal fat      | 1.1 ± 0.06              | 0.6 ± 0.1                 | 45            | 0.006       |
| Mesenteric fat    | 0.6 ± 0.07              | 0.3 ± 0.08                | 50            | 0.05        |
| EDL muscle        | 1.4 ± 0.1               | 0.6 ± 0.1                 | 57            | 0.004       |
| Soleus muscle     | 1.0 ± 0.1               | 0.5 ± 0.04                | 50            | 0.002       |

Levels of GR mRNA in brain were measured by \textit{in situ} mRNA hybridization using a cRNA probe complementary to exons 5–9 of GR, absent from mRNA encoding the GR\textsuperscript{geo} fusion protein. Levels of full-length GR mRNA in peripheral tissues were measured by qPCR using a primer-probe set spanning exons 5–6. Student’s \(t\) test was performed for comparisons between genotypes (\(n=6/\)group). Significance was set at \(P<0.05\). AU, arbitrary units; CA, cornu ammonis; DG, dentate gyrus; PVN, paraventricular nucleus of the hypothalamus; EDL, extensor longus digitalis.
POMC mRNA levels, only in GR+/+ mice (LF- vs. HF-fed GR+/- mice: 0.7 ± 0.1 and 1.3 ± 0.3, respectively, P=0.3; LF- vs. HF-fed GRβgeo/+ mice: 0.9 ± 0.2 and 0.9 ± 0.2, respectively, n=5/group). Strikingly, and similar to basal plasma corticosterone levels, adrenal weight was significantly higher in LF-fed GRβgeo/+ mice compared with their GR+/- littermates, but, in contrast to GR+/- mice, in which adrenal weight was increased by the HF diet, the HF diet had no effect on adrenal weight in GRβgeo/+ mice (Fig. 4C). Adrenal morphology also differed between genotypes, and this was particularly apparent on the LF diet (Fig. 5). The morphology suggests stimulation of corticosterone-producing cells of GRβgeo/+ mice in the zona fasciculata and glomerulosa, respectively. In adrenal glands from GRβgeo/+ mice, the columns of endocrine cells in the zona fasciculata are longer than in GR+/- mice, with individual cells having homogeneous eosinophilic cytoplasm (Fig. 5A-D). The zona glomerulosa of GRβgeo/+ adrenal glands appears thicker than in GR+/- mice, with more glomeruli evident and containing hypertrophied epithelium (Fig. 5C, D). This adrenal phenotype was also apparent in mice fed the HF diet (Fig. 5E, F), but the morphological differences between LF and HF groups were more pronounced in GR+/- mice than in GRβgeo/+ mice (Fig. 5). Measurements of adrenal cell size in the different zones (glomerulosa, fasciculata, and reticularis) did not differ between genotypes (data not shown), indicating that the larger adrenal glands in GRβgeo/+ mice reflect hyperplasia rather than cellular hypertrophy.
GR<sup>geo</sup/>mice have normal adipose tissue distribution and glucose homeostasis but higher liver triglyceride levels on HF diet

Although GR polymorphisms in humans are associated with alterations in body weight and/or composition (reviewed in ref. 14), there were no differences in body weight (Fig. 6A) or food intake (data not shown) between GR<sup>geo</sup/>mice and their GR<sup>+</sup/> littermates, on either the LF or HF diet. There were also no differences between genotypes on either diet in weights of adipose tissue (epididymal, inguinal, and mesenteric) and muscle (extensor digitorum longus) or in plasma levels of insulin, NEFAs, and triglyceride (Table 2). However, while the HF diet caused the expected increase in liver triglyceride levels in both genotypes, levels were higher in HF-fed GR<sup>geo</sup/> than in GR<sup>+</sup/>mice (Table 2). Both genotypes fed the LF diet showed similar responses in GTTs with an identical impaired response in mice fed HF diet (Fig. 6B).

**GR<sup>geo</sup/>mice are hypertensive and show an attenuated blood pressure response to HF diet**

In LF-fed mice, systolic blood pressure was significantly elevated (8 mmHg) in GR<sup>geo</sup/> mice compared with their GR<sup>+</sup/> littermates (Fig. 7). Furthermore, blood pressure was increased in both genotypes by the HF diet, although interestingly the magnitude of the increase was lower in GR<sup>geo</sup/> mice (8 mmHg) than in GR<sup>+</sup/> mice (14 mmHg), resulting in no difference in blood pressure between genotypes in mice fed the HF diet (Fig. 7).

**TABLE 2. Similar regional adiposity, lean mass, and plasma insulin and lipid levels in GR<sup>geo</sup/> and GR<sup>+</sup/>mice**

| Variable            | LF diet        | GR<sup>geo</sup/> | GR<sup>+</sup/> | HF diet        | GR<sup>geo</sup/> | GR<sup>+</sup/> |
|---------------------|----------------|-------------------|----------------|----------------|-------------------|----------------|
| Epididymal fat mass |                |                   |                |                |                   |                |
| % Body weight       | 2.08 ± 0.36    | 1.66 ± 0.38       | 4.42 ± 0.22*** | 4.90 ± 0.38*** |                   |                |
| Absolute wt (mg)    | 657 ± 140      | 489 ± 126         | 1972 ± 110     | 2476 ± 285     |                   |                |
| Inguinal fat mass   |                |                   |                |                |                   |                |
| % Body weight       | 1.79 ± 0.31    | 1.36 ± 0.07       | 4.22 ± 0.27*** | 4.5 ± 0.35***  |                   |                |
| Absolute wt (mg)    | 559 ± 119      | 387 ± 23          | 1914 ± 186     | 2230 ± 274     |                   |                |
| Mesenteric fat mass |                |                   |                |                |                   |                |
| % Body weight       | 1.31 ± 0.15    | 1.07 ± 0.07       | 2.16 ± 0.13**  | 2.00 ± 0.17**  |                   |                |
| Absolute wt (mg)    | 408 ± 67       | 301 ± 31          | 979 ± 92       | 984 ± 121      |                   |                |
| Brown adipose mass  |                |                   |                |                |                   |                |
| % Body weight       | 0.38 ± 0.03    | 0.38 ± 0.03       | 0.62 ± 0.05**  | 0.79 ± 0.05**  |                   |                |
| Absolute wt (mg)    | 118 ± 15       | 107 ± 8           | 282 ± 31       | 381 ± 34       |                   |                |
| Muscle (EDL) mass   |                |                   |                |                |                   |                |
| % Body weight       | 0.24 ± 0.02    | 0.22 ± 0.02       | 0.16 ± 0.01**  | 0.15 ± 0.01**  |                   |                |
| Absolute wt (mg)    | 70.3 ± 4       | 60.8 ± 5          | 68.6 ± 3       | 68.8 ± 3       |                   |                |
| Plasma insulin (ng/ml) | 0.2 ± 0.01 | 0.3 ± 0.1         | 0.9 ± 0.2*     | 1.0 ± 0.2*     |                   |                |
| Plasma NEFA (mEq/l) | 0.8 ± 0.1      | 0.6 ± 0.1         | 0.65 ± 0.1     | 0.8 ± 0.1      |                   |                |
| Plasma TG (mmol/l)  | 0.44 ± 0.05    | 0.37 ± 0.06       | 0.52 ± 0.03    | 0.47 ± 0.04    |                   |                |
| Liver TG (µmol/g)   | 5.4 ± 0.7      | 8.0 ± 1.6         | 14.8 ± 0.7***  | 18 ± 0.8***    |                   |                |

Plasma NEFA, plasma triglyceride (TG), and plasma insulin levels were measured after 24 h fast. *P < 0.05; **P < 0.01; ***P < 0.001 vs. LF diet. Values in italics indicate a significant difference between genotypes. n = 8/group.
**GR<sup>geo/+</sup></sup> mice have an activated RAAS**

Increased systolic blood pressure in GR<sup>geo/+</sup> mice was associated with a 2-fold increase in plasma renin activity, which was unaffected by diet in either genotype, remaining higher in GR<sup>geo/+</sup> mice (Fig. 8A). Similarly, plasma aldosterone levels were increased in GR<sup>geo/+</sup> mice, irrespective of diet (Fig. 8B). Plasma angiotensinogen levels were comparable between LF diet-fed GR<sup>geo/+</sup> and GR<sup>+/+</sup> mice but were markedly increased (3-fold) by the HF diet only in GR<sup>geo/+</sup> mice (Fig. 8C). Hepatic angiotensinogen mRNA levels mirrored the pattern of plasma angiotensinogen (Fig. 8E). Increased adipose tissue expression of angiotensinogen, although much lower than hepatic expression, has been postulated to contribute to or even underlie hypertension in diet-induced obesity (38). However, although we observed higher expression of angiotensinogen mRNA in epididymal adipose tissue of LF-fed GR<sup>geo/+</sup> mice compared with GR<sup>+/+</sup> mice, levels were lower in HF-fed mice and did not differ between genotypes (Fig. 8E). Neither kidney nor adrenal renin mRNA levels differed between genotypes on the LF diet nor were they altered by the HF diet (Table 3). Similarly, renal 11β-HSD2 and MR mRNA levels, both critical determinants of blood pressure (39), were identical between genotypes and they did not differ with diet (Table 3). Thus, activation of the RAAS in GR<sup>geo/+</sup> mice is likely to underlie their hypertension but does not account for changes in blood pressure after the HF diet. The activation of RAAS in GR<sup>geo/+</sup> mice was not accompanied by grossly abnormal kidney function, since kidney morphology was similar in both genotypes (data not shown).

**DISCUSSION**

Although GR polymorphisms are associated with hypertension and differences in body mass index and body composition in humans, few animal studies have addressed the effects of altered GR density on body weight and composition and none have examined the effect on metabolic adaption to the HF diet. Here we found that reduced tissue GR density has no effect on body weight, adipose tissue distribution or glucose homeostasis, basally, on a chow diet (unpublished observations) or an LF diet or after an HF diet. However, like most humans heterozygous for mutations in the GR gene, GR<sup>geo/+</sup> mice have an activated HPA axis and, as we have now shown, elevated blood pressure. Interestingly, GR<sup>geo/+</sup> mice failed to show the full extent of the HPA and blood pressure adaption to the HF diet seen in control mice, suggesting that tissue GR density limits the adaptive response to chronic dietary stress.

As expected, GR<sup>geo/+</sup> mice display a hyperactive HPA axis, with elevated plasma corticosterone and larger adrenal glands. The latter results from hyperplasia rather than cellular hypertrophy, since adrenal cell size was not different in GR<sup>geo/+</sup> mice in any of the zones. This is consistent with previous data showing increased plasma corticosterone levels in mice with reduced GR density (11, 18), although measurements in these previous studies are likely to have been stress levels. It differs, however, from a previous study in mice heterozygous for a null allele of GR (GR<sup>sel/+</sup>) in which nonstressed plasma corticosterone levels, both morning and evening, were the same as in control mice, although the mice did show an increased plasma corticosterone response to stress (40), similar to GR<sup>geo/+</sup> mice (our unpublished data). The discrepancy in nonstressed plasma corticosterone levels between the two models may be related to environmental or strain differences: here, backcrossed to C57BL/6J for five generations, whereas Ridder et al. (40) examined F<sub>1</sub> offspring of a C57BL/6JX FVB/N cross. We (41) have noted higher basal plasma corticosterone levels in FVB/N mice.

**GR<sup>geo/+</sup> mice have completely normal body weight, regional adiposity, and lean mass.** Glucose homeostasis (measured by fasting plasma glucose) and insulin levels, as well as glucose tolerance, were also entirely normal in GR<sup>geo/+</sup> mice. This metabolic phenotype contrasts with the obesity seen in chow-fed transgenic mice with globally reduced GR density because of antisense GR RNA expression (18, 19). These mice, similar to GR<sup>geo/+</sup> mice, also show an activated HPA axis (18). The reason for the discrepancy between these two models, each with reduced GR and elevated corticosterone, is not clear but may relate to the unknown effects of the GR transgene in the antisense GR-expressing mice in which knockdown of the GR is achieved through use of a neurofilament gene promoter, which may be differentially expressed within the central nervous system. Mice with a conditional deletion of the GR gene in neurons have very high plasma corticosterone levels due to a lack of negative feedback at the hypothalamus (42). However, these mice have normal peripheral GR levels, are growth retarded, and have proportionately more body fat before weaning, but less...
after (20). In GR<sup>geo/+</sup> mice, reduced peripheral GR density may compensate for the increased plasma corticosterone levels, normalizing body fat distribution. The lack of effect of genotype on glucose homeostasis is not surprising, as selective GR gene deletion in hepatocytes showed that GR is essential for glucose homeostasis only after prolonged fasting or in a diabetic state (21). Moreover, antisense GR mice also show normal glucose homeostasis (19).

Like most humans heterozygous for inactivating mutations in the GR gene (43), GR<sup>geo/+</sup> mice are hypertensive. In GR<sup>geo/+</sup> mice, the hypertension results from elevated activity of the renin-angiotensin-aldosterone system. Plasma aldosterone levels were increased in GR<sup>geo/+</sup> mice, as was plasma renin activity. The latter, however, was not associated with increased expression of renin mRNA in either the kidney or adrenal gland. A similar discrepancy between plasma renin and mRNA levels has previously been reported and postulated to be due to the more rapid secretion and turnover of newly synthesized renin compared with the long half-life of renin mRNA (44). Alternatively, renin clearance may be increased in GR<sup>geo/+</sup> mice. Despite reduced GR density and elevated plasma corticosterone levels in GR<sup>geo/+</sup> mice, there were no compensatory changes, at least in the kidney, in the expression of MR or 11β-HSD2, which protects MR from illicit occupation by glucocorticoids (39). Similarly, MR levels in the brain are normal in mice with forebrain-specific deletion of GR (45), suggesting that MR density is not altered to compensate for reduced GR expression. The increased plasma aldosterone levels in GR<sup>geo/+</sup> mice were consistent with the adrenal histopathology, suggesting high synthetic activity of aldosterone producing cells. This was predicted from the phenotype of GR<sup>hypo/hypo</sup> mice, which have elevated levels of aldosterone synthase in

![Figure 8. Activation of the RAAS in GR<sup>geo/+</sup> mice. Plasma renin activity (A), plasma aldosterone concentration (B), plasma angiotensinogen concentration (C), epididymal fat angiotensinogen (AGT) mRNA levels (D), and hepatic AGT mRNA levels (E) in LF-fed GR<sup>+/+</sup> mice (white bars), HF-fed GR<sup>+/+</sup> mice (hatched bars), LF-fed GR<sup>geo/+</sup> mice (black bars), and HF-fed GR<sup>geo/+</sup> mice (gray bars). For plasma measurements, n = 5/group. For mRNA levels, n = 8–9/group. *†P < 0.05; **††P < 0.01; ***†††P < 0.001.](image)

**Table 3.** Similar expression of mRNAs encoding renin, 11β-HSD2, and MR in GR<sup>geo/+</sup> and GR<sup>+/+</sup> mice

| mRNA     | Tissue | LF diet | HF diet |
|----------|--------|---------|---------|
|          |        | GR<sup>+/+</sup> | GR<sup>geo/+</sup> | GR<sup>+/+</sup> | GR<sup>geo/+</sup> |
| Renin    | Kidney | 0.87 ± 0.07 | 0.95 ± 0.20 | 0.83 ± 0.09 | 0.75 ± 0.06 |
| Adrenal  |        | 0.98 ± 0.20 | 1.0 ± 0.10  | ND        | ND          |
| 11β-HSD2 | Kidney | 0.85 ± 0.03 | 0.86 ± 0.09 | 0.94 ± 0.04 | 0.77 ± 0.09 |
| MR       | Kidney | 0.80 ± 0.08 | 0.96 ± 0.20 | 0.98 ± 0.07 | 0.99 ± 0.10 |

Levels of specific mRNAs were measured by qPCR and are expressed in arbitrary units. There were no significant effects of either genotype or diet upon levels of any of the mRNAs measured (n=6/group).
the zona glomerulosa (11), and is probably a consequence of elevated plasma ACTH levels in these mice. Indeed, Pomc/geo mice, which lack circulating ACTH, have small adrenal glands and dramatically reduced plasma aldosterone levels (46).

HF diets chronically stress the HPA axis in rodents, increasing basal corticosterone levels and causing adrenal enlargement (35, 47), although negative feedback efficiency is unaffected by HF diet (35). Consistent with this, in GRgeo/+ mice, the HF diet from weaning increased adrenal size and increased morning plasma corticosterone levels. In contrast, in GRgeo/geo mice, the HF diet had no effect on either of these parameters. Thus, differences between genotypes in these parameters were eliminated by the diet. Evening (nonstressed) plasma corticosterone levels were unaffected by the HF diet or the GR density of GENS5 mice. These results suggest that the HPA axis response to the HF diet is limited by tissue GR density, this adaptive response being abolished by a 50% reduction in GR levels.

Surprisingly, given the role of glucocorticoids in energy homeostasis (2), GRgeo/geo mice showed an entirely normal body weight gain, altered glucose homeostasis and insulin sensitivity on the HF diet, and did not differ from GR+/+ controls. Similarly, mice of both genotypes fed the HF diet doubled their hepatic accumulation of triglyceride compared with mice on the control diet, although hepatic triglyceride levels were significantly higher in HF-fed GRgeo/geo mice than in GR+/+ mice. Although plasma NEFAs did not differ between LF- and HF-fed mice, these were measured after a 24 h fast, when NEFAs release from adipose tissue constitutes the major energy source and is maximal. Glucocorticoids increase hepatic lipogenesis and secretion (48–50). Furthermore, the consequences of altered glucocorticoid status on hepatic triglyceride metabolism are dependent on diet composition (51), but only when the diet is fed ad libitum (52). Thus, if lipid flux is low, when animals are maintained on an LF diet or with the restricted HF diet, then glucocorticoids minimally affect hepatic triglyceride storage and secretion (52). Whether the increased hepatic triglyceride levels in GRgeo/geo mice are a consequence of the elevated peak corticosterone levels in these mice (morning levels did not differ after the HF diet) or a direct consequence of reduced hepatic GR density remains to be determined. 11β-HSD1 levels are unlikely to contribute to elevated hepatic triglycerides, since adipose or liver mRNA levels were unaltered (unpublished observations).

Blood pressure was increased by the HF diet in both genotypes, consistent with previous data (36, 37, 53). However, the magnitude of the HF-induced increase in blood pressure was smaller in GRgeo/geo mice so that blood pressure in HF-fed GRgeo/geo mice did not differ from that in HF-fed GR+/+ mice. The mechanism underlying the blood pressure increase with the HF diet remains unclear but may differ between genotypes. In GRgeo/geo mice, further RAAS activation may contribute. Plasma angiotensinogen levels were increased by the HF diet in GRgeo/geo mice, probably as a result of increased hepatic expression of angiotensinogen, but were unaffected in GR+/+ mice in which the diet-induced increase in blood pressure was greater. The HF diet had no effect on plasma aldosterone levels or plasma renin activity in either genotype. Thus, activation of the systemic RAAS by the HF diet in GRgeo/geo mice is ruled out. Elevated plasma glucocorticoid levels drive hypertension in both humans and rodents (1, 54). However, the underlying mechanisms remain controversial but are likely to include changes in the sympathetic nervous system. In this regard, it is of note that adrenal catecholamines, implicated in the HF diet-induced hypertension (53), are reduced in GRgeo/geo mice (11, 34). It will be of interest, in future work, to determine whether sympathetic nervous system activity is altered in GRgeo/geo mice.

In humans, polymorphisms in the GR gene associated with hypersensitivity or hyposensitivity to glucocorticoids in a dexamethasone suppression test have been variably associated with differences in body mass index, waist-hip ratio, altered body composition, and metabolic parameters (reviewed in ref. 14) as well as differences in blood pressure (55–59). However, the association studies (14) are controversial and may depend on age, gender, and ethnic background. The data reported here provide insights into that heterogeneity. In our model of glucocorticoid resistance due to ~50% reduction in GR density, the observed physiological differences between genotypes depend on environment. Thus, several of the differences between genotypes are abolished or reduced after the HF diet. If the same applies in humans, then (for example) obesity or other chronic stress may mask differences in blood pressure between populations with different GR polymorphisms.

Glucocorticoids form part of important homeostatic control mechanisms that are critical in adapting to environmental challenges. Thus, they maintain stability through change over time (allostasis; reviewed in refs. 60, 61). GRgeo/geo mice do not show the normal adaptations to the HF diet; they have an attenuated blood pressure response and do not show the alterations in adrenal size and HPA axis activity seen in WT mice. Consequently, basal corticosterone levels are normalized between genotypes after the HF diet. Moreover, GRgeo/geo mice accumulated more hepatic triglyceride, possibly because of inadequate adaption to excessive dietary fat/energy intake and the need to protect vital organs. Continued excessive lipid accumulation in the liver may, in turn, lead to insulin resistance and metabolic disease. GR density is likely to be limiting in other systems in adaption to environmental stressors. Mice heterozygous for inactivating mutations in GR show reduced coping behavior in a mouse correlate of depression, with compromised indicators of neural plasticity (40), whereas overexpression of GR results in resistance to stress and robust protection against endotoxins. These adaptive or “remodeling” effects of glu-
corticoids in the adult response to the HF diet are reminiscent of the prenatal “programming” effects of glucocorticoids (reviewed in ref. 62) where exposure of the late fetus to glucocorticoids permanently programs tissue systems throughout life, including blood pressure, glucose/lipid homeostasis, and tissue GR density itself. It will be of great interest to determine whether GR density influences the prenatal programming effects of glucocorticoids in the same way that it alters the postnatal blood pressure and HPA adaptions to a chronic HF diet.

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