Susceptibility to Diet-Induced Hepatic Steatosis and Glucocorticoid Resistance in FK506-Binding Protein 52-Deficient Mice

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Although FK506-binding protein 52 (FKBP52) is an established positive regulator of glucocorticoid receptor (GR) activity, an *in vivo* role for FKBP52 in glucocorticoid control of metabolism has not been reported. To address this question, FKBP52$^{+/-}$ mice were placed on a high-fat (HF) diet known to induce obesity, hepatic steatosis, and insulin resistance. Tissue profiling of wild-type mice showed high levels of FKBP52 in the liver but little to no expression in muscle or adipose tissue, predicting a restricted pattern of FKBP52 effects on metabolism. In response to HF, FKBP52$^{+/-}$ mice demonstrated a susceptibility to hyperglycemia and hyperinsulinemia that correlated with reduced insulin clearance and reduced expression of hepatic CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1), a mediator of clearance. Livers of HF-fed mutant mice had high lipid content and elevated expression of lipogenic genes (peroxisome proliferator-activated receptor γ, fatty acid synthase, and sterol regulatory element-binding protein 1c) and inflammatory markers (TNFα). Interestingly, mutant mice under HF showed elevated serum corticosterone, but their steatotic livers had reduced expression of gluconeogenic genes (phosphoenolpyruvate carboxy kinase, glucose 6 phosphatase, and pyruvate dehydrogenase kinase 4), whereas muscle and adipose expressed normal to elevated levels of glucocorticoid markers. These data suggest a state of glucocorticoid resistance arising from liver-specific loss of GR activity. Consistent with this hypothesis, reduced expression of gluconeogenic genes and CEACAM1 was observed in dexamethasone-treated FKBP52-deficient mouse embryonic fibroblast cells. We propose a model in which FKBP52 loss reduces GR control of gluconeogenesis, predisposing the liver to steatosis under HF-diet conditions attributable to a shunting of metabolism from glucose production to lipogenesis. **(*Endocrinology* 151: 3225–3236, 2010)**

As antagonists to insulin, glucocorticoid hormones (GCs) play an important role in diabetes and metabolic syndrome. A variety of reports has linked overstimulation by GCs to various aspects of metabolic syndrome: central obesity, myopathy, fatty liver (steatosis), hypercholesterolemia, hyperglycemia, and insulin resistance (for review, see Ref. 1). Indeed, an evolving school of thought ties chronic, stress-induced GC levels to the high

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-1158 Received September 25, 2009. Accepted March 29, 2010.
First Published Online April 28, 2010
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Abbreviations: AR, Androgen receptor; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; CoA, coenzyme A; Cyp40, cyclophilin-40; Dex, dexamethasone; ER, estrogen receptor; FAS, fatty acid synthase; FFA, free fatty acid; FKBP, FK506-binding protein; G6Pase, glucose 6 phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, glucocorticoid hormone; GR, glucocorticoid receptor; HF, high fat; HPA, hypothalamus-pituitary-adrenal; Hsp90, heat shock protein 90; MEF, mouse embryonic fibroblast; PDC, pyruvate dehydrogenase complex; PDK4, pyruvate dehydrogenase kinase 4; PEPCK, phosphoenolpyruvate carboxy kinase; PP5, protein phosphatase 5; PPARγ, peroxisome proliferator-activated receptor γ; PR, progesterone receptor; PSA, prostate-specific antigen; RD, regular diet; SREBP1c, sterol regulatory element-binding protein 1c; TG, triglyceride; TPR, tetratricopeptide repeat; WAT, white adipose tissue; WT, wild type.

incidence of type 2 diabetes (2). However, the widespread and potent side effects of GC ligands have precluded the use of GC antagonists in the treatment of metabolic disorders. Thus, new and selective targets of GC action are needed. A body of work has suggested that tetratricopeptide repeat (TPR) proteins that interact with the glucocorticoid receptor (GR) may serve this purpose.

The GR is a hormone-activated transcription factor controlling specific gene expression (3, 4). Like other members of the steroid receptor family, GR exists as a large heterocomplex in the hormone-free state containing heat shock protein 90 (Hsp90) and one of several TPR proteins: FK506-binding proteins (FKBP51 and FKBP52), FKBP-like, cyclophilin-40 (Cyp40), and protein phosphatase 5 (PP5) (5). Of these, FKBP51 and FKBP52 have been the most studied with respect to GR action. FKBP52 and FKBP51 exert differential effects on GR's binding to hormone and transcriptional activation function, with FKBP51 being inhibitory and FKBP52 stimulatory (6–8). *In vivo* studies of FKBP52 have been done recently, but these have shown that FKBP52 plays important roles in fertility by contributing to progesterone receptor (PR) control of uterine receptivity to implantation (9, 10) and to androgen receptor (AR) control of male sexual development (11, 12). To date, no studies have been reported on the contribution of FKBP52 to GR-controlled physiology.

In mammals, homeostatic control of blood glucose is achieved through the counter-regulatory actions of insulin, glucagon, catecholamines, and glucocorticoids. In general, the major role of GCs is to promote hepatic gluconeogenesis in response to long-term fasting or other forms of prolonged stress (1, 13). To achieve this, GCs will also mobilize free fatty acids (FFAs) from peripheral adipose and amino acids from muscle. In liver, the principal targets of GC-induced gluconeogenesis are phosphoenolpyruvate carboxy kinase (PEPCK) and glucose 6 phosphatase (G6Pase) (14–16). To ensure that pyruvate is not shunted to lipogenic pathways, GCs also induce pyruvate dehydrogenase kinase 4 (PDK4), which inhibits the ability of pyruvate dehydrogenase to convert pyruvate to acetylcoenzyme A (CoA) (17).

Although prolonged fasting may be the best understood mechanism eliciting the metabolic effects of GCs, more subtle effects of GCs on metabolism attributable to circadian fluctuations or chronic stress are only now being appreciated. New studies suggest that overstimulation by GCs leads to the diverse symptoms of metabolic syndrome, including insulin resistance (1). The first demonstration of this syndrome was in patients with Cushing's disease (excess GC production), yet moderate to severe cases of the syndrome can occur in response to chronic induction of GC secretion (18, 19). The process by which GCs induce insulin resistance is likely to be multifaceted. Well-known examples are seen in muscle and adipose tissue in which GCs cause down-regulation of insulin signaling (20–22). GCs can also induce a fatty liver state. Whole animal studies have demonstrated that GCs lead to increased hepatic triglyceride (TG) synthesis and lipid accumulation (23). Studies in isolated hepatocytes indicate that GCs can play a direct role to stimulate lipogenesis (24–26).

Although fatty liver reduces hepatic sensitivity to insulin (27–29), an additional mechanism of impaired insulin clearance by the liver has been uncovered involving the insulin receptor substrate carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). Hepatic CEACAM1 promotes insulin removal from blood via receptor-mediated endocytosis and degradation (30–32). Importantly, reduction of hepatic CEACAM1 or its function leads to hyperinsulinemia, fatty liver, and insulin resistance (33–36). Interestingly, Ceacam1 promoter activity is positively regulated by GC agonists (37).

In this work, the contribution of FKBP52 to the GR-insulin axis was explored by subjecting FKBP52 mutant mice to a high-fat (HF) feeding regimen known to induce insulin resistance. Given the known contribution of GCs to fatty liver and reduced GR activity in FKBP52-deficient cells, we hypothesized a phenotype of reduced susceptibility to metabolic disorders, such as insulin resistance. Surprisingly, FKBP52-deficient mice fed HF demonstrated elevated plasma levels of corticosterone, insulin, and glucose, along with exaggerated hepatic steatosis and reduced CEACAM1-mediated insulin clearance in liver. Because hepatic levels of GR-induced gluconeogenic enzymes were also reduced, we propose a model in which reduced gluconeogenesis in the face of an HF diet leads to shunting of carbons to lipogenic pathways, with eventual induction of a fatty liver state.

Materials and Methods

Animals

The generation of FKBP52 null mice has been described previously (10). The original FKBP52 strain was established on a mixed C57BL/6J:129SvEv genetic background and yielded a high rate of embryonic lethality among null animals. FKBP52 null mice independently generated by Smith and colleagues (9, 11) also showed low viability of null embryos. The current colony, maintained on the same mixed background, shows nearly 100% embryonic lethality for FKBP52$^{-/-}$ animals but a normal Mendelian ratio of FKBP52$^{+/-}$ newborns. Hence, FKBP52 heterozygote mice were used in this study. Animals were obtained by double heterozygote mating, with wild-type (WT) littermates used as controls. All experiments were performed on male mice,

2 months of age. Animals were housed in a temperature-controlled environment with a 12-h light, 12-h dark cycle. All procedures were approved by the Institutional Animal Care and Utilization Committee of the University of Toledo.

Diet and metabolic analyses

The breeding colony was maintained on standard chow [regular diet (RD)] containing 12 kcal% fat *ad libitum*. Experimental WT and FKBP52$^{+/-}$ mice were separated into two groups of 8-wk-old males. One group was fed (*ad libitum*) a RD for 4 wk, and the second was fed (*ad libitum*) a HF diet containing 45 kcal% fat (catalog no. D12451; Research Diets, New Brunswick, NJ). To eliminate the confounding effects of fasting on GC responses, all tissues and blood samples were collected from animals under random-fed conditions. The HF regimen chosen is known to induce borderline to moderate steatosis and insulin resistance in most strains of mice, without stimulating hepatic inflammation (35).

Whole venous blood was drawn from retro-orbital sinuses. Blood glucose levels were measured using a glucometer (Accucheck Aviva; Roche Diagnostics, Indianapolis, IN). Corticosterone (MP Biomedicals, Irvine, CA), plasma insulin, and C-peptide levels (Linco Research, St. Charles, MO) were measured by RIA. Plasma FFAs (Wako Bioproducts, Richmond, VA) and TGs (Pointe Scientific, Canton, MI) were measured by colorimetric assay.

Gel electrophoresis and Western blotting

Extracts were prepared by homogenization of tissue samples in lysis buffer [150 mM NaCl, 50 mM HEPES (pH 7.6), 0.02% sodium azide, 1% Triton X-100, and freshly added protease inhibitors]. Homogenized samples were rotated at 4 C for 90 min, followed by centrifugation at 20,000 × *g* for 30 min. Supernatant was allocated based on equal protein content and resolved on 10% polyacrylamide SDS gels, as described by Laemmli (38). Proteins were transferred to Immobilon polyvinylidene difluoride membrane. Specific primary antibodies were used to probe for each protein tested: GR (FiGR monoclonal antibody was a gift from Jack Bodwell, Dartmouth Medical School, Hanover, NH), FKBP51, FKBP52, Hsp90, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA), Cyp40 (Affinity Bioreagents, Golden, CO), PP5 (gift from Michael Chinkers, University of South Alabama, College of Medicine, Mobile, AL), ApoB48/100 (Millipore Bioscience Research Reagents, Temecula, CA), fatty acid synthase (FAS) (36), and microsomal triglyceride transfer protein-1 (MTP1) (BD Biosciences, San Jose, CA). The blots were incubated with appropriate secondary antibodies, developed by enhanced chemiluminescence, and quantified by densitometry.

Oil Red O staining and tissue TG content

Frozen liver samples were sliced with a cryostat to 10 μm thickness. The sections were stained with Oil Red O (Sigma, St. Louis, MO) and counterstained with hematoxylin (Thermo Fisher Scientific, Waltham, MA). Hepatic TG content was measured by extracting lipid from homogenized samples with $CHCl_3$:CH_3OH (2:1, vol/vol). The samples were dried under nitrogen and resuspended using ethanol. TG levels were measured by colorimetric assay (Pointe Scientific).

Transient transfection and luciferase reporter assays

Mouse embryonic fibroblast (MEF) cells were made from WT and FKBP52$^{-/-}$ embryos at d 13.5 of gestation, as described previously (10). MEF cells were cultured in DMEM containing 10% charcoal-stripped calf serum to 90% confluence in six-well plates, followed by transient transfection with pGRE2EIB-Luc or pPEPCK-Luc (gift from Dr. Darryl Granner, Vanderbilt Medical Center, Nashville, TN) or prostate-specific antigen (PSA)-Luc reporter constructs using Lipofectamine 2000 (Invitrogen). To normalize transfection efficiency, cytomegalovirus-driven galactosidase reporter was cotransfected. Twenty-four hours after transfection, cells were treated with hormone and harvested after an additional 24 h. Luciferase assays were performed using a kit from Promega (Madison, WI).

Real-time PCR

Total RNA was isolated from MEF cells or tissues using 5-Prime PerfectPure RNA Cell kit (Thermo Fisher Scientific) according to the instructions of the manufacturer. Total RNA concentration and purity was determined by measuring absorbance at 260/280 nm and confirmed on an RNA denaturing formaldehyde gel. Purified RNA (1 μg) was used to produce cDNA using a first-strand synthesis kit (Roche Applied Science, Indianapolis, IN). PCR amplification of the cDNA was performed by quantitative real-time PCR (Applied Biosystems, Foster City, CA) using qPCR Core kit for SYBR Green I (Applied Biosystems). Primers were designed using Primer Express 3.0 software (Applied Biosystems). For normalization, in separate reactions, primers were used to amplify 18S mRNA. To study whether genomic sequences were amplified, a control sample was used in which no reverse transcriptase was added. The thermocycling protocol consisted of 10 min at 95 C, 40 cycles of 15 sec at 95 C, 30 sec at 61 C, and 20 sec at 72 C and finished with a melting curve ranging from 60 to 95 C to allow distinction of specific products.

Statistical analysis

Data were analyzed with Prism 5 (GraphPad Software, San Diego, CA) using unpaired *t* tests. *P* values <0.05 were considered statistically significant.

Results

Optimal GR activity at metabolic genes requires FKBP52

To evaluate the role of FKBP52 on GR control of metabolism, gene expression was assayed in WT and FKBP52$^{-/-}$ MEF cells treated with the glucocorticoid agonist Dex. Before treatment, Western blot analysis revealed higher protein levels of GR in FKBP52$^{-/-}$ cells (Fig 1A). This difference was confirmed by quantitative real-time PCR (Fig. 1B), suggesting that a compensation mechanism may exist at the level of GR transcription. Higher GR expression, however, did not correlate with greater activity, because reduced responsiveness to Dex was seen in FKBP52$^{-/-}$ cells transfected with pGRE2EIB-Luc (data

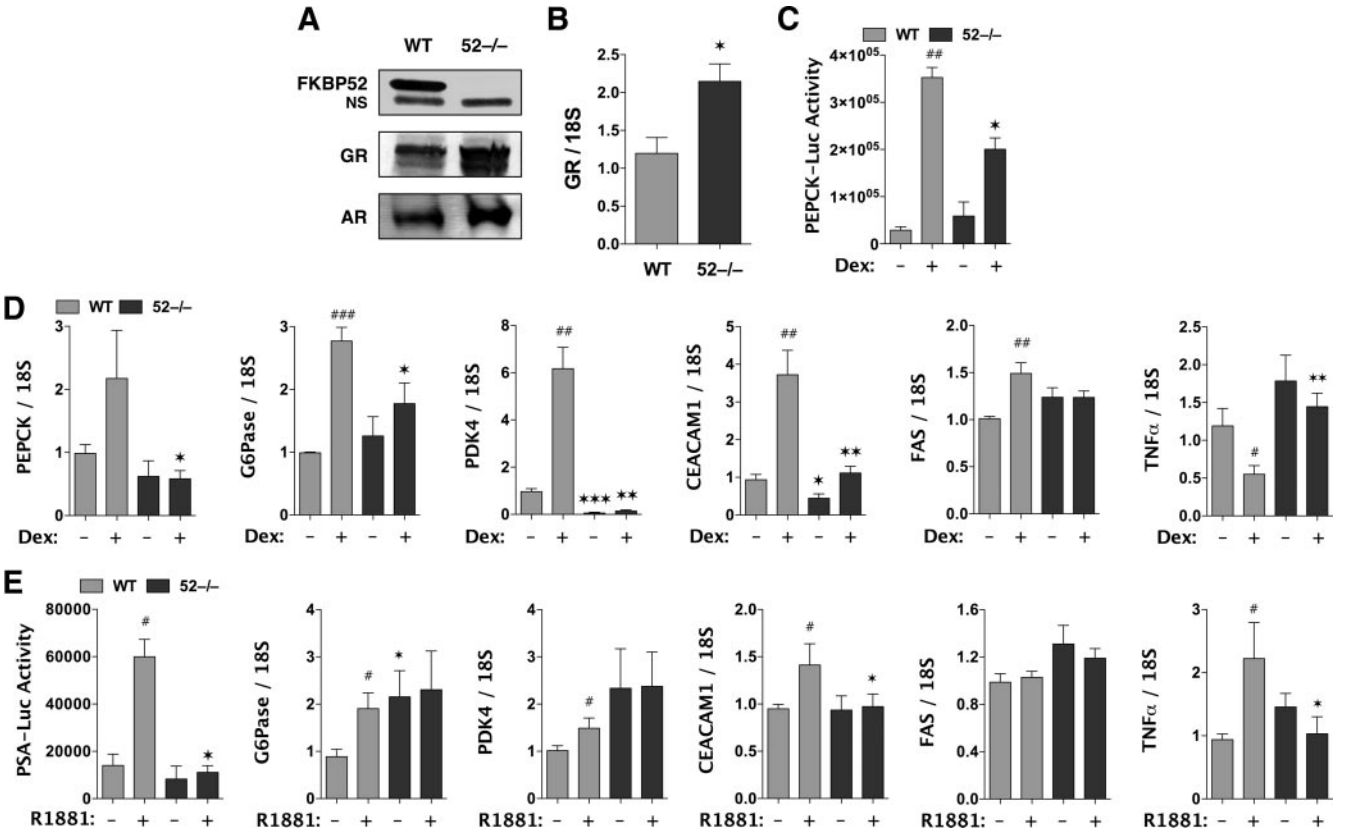

FIG. 1. Up-regulation of GR protein but reduced GR activity at gluconeogenic genes in FKBP52-deficient MEFs. GR expression in WT and FKBP52$^{-/-}$ MEF cells was measured by Western blotting (A; n = 3) and real-time PCR (B; n = 5). GR activity in WT and FKBP52$^{-/-}$ cells was measured by transient transfection with PEPCK-Luc (C; n = 3), followed by treatment with 1 μM Dex for 24 h or by real-time PCR analysis of the indicated endogenous genes (D, n = 4–6) after treatment with 100 nM Dex for 2 h. To test for the contribution of FKBP52 to AR activity, WT and FKBP52$^{-/-}$ MEF cells were stably transfected with AR and analyzed by Western blotting (A) and transient transfection with PSA-Luc reporter or real-time PCR analysis of the indicated endogenous genes (E; n = 3–6) after treatment with 10 nM R1881 for 2 h. All values represent means ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* WT). #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (*vs.* hormone-free control).

not shown) and PEPCK-Luc reporter constructs (Fig. 1C). This result was confirmed by real-time PCR analysis (Fig. 1D). When treated with Dex, expression levels of three gluconeogenic genes (PEPCK, G6Pase, and PDK4) were induced in WT cells but only minimally up-regulated or down-regulated in FKBP52 null cells. GR activity in FKBP52$^{-/-}$ cells was also reduced at CEACAM1, the mediator of hepatic insulin clearance (34), and showed a trend to reduced expression of FAS, a major target of GC-induced lipogenesis (39). Last, it was found that Dex suppression of TNFα was abrogated in mutant MEF cells, resulting in high levels of the inflammatory cytokine even under basal (no Dex) conditions. Together, these results show that FKBP52 is required for the optimal GR activity at key metabolic genes.

Because of recent work suggesting that AR may control lipid and glucose metabolism in the liver (40), we also tested for FKBP52 involvement in AR regulation of metabolic genes (Fig. 1E). FKBP52$^{-/-}$ cells had reduced responsiveness to androgen agonist R1881 at the PSA-Luc reporter construct, as reported previously (12). In contrast to glucocorticoids, androgens can increase expression of TNFα, and this ability was also reduced in FKBP2 null cells. Interestingly, although R1881 moderately increased expression of PEPCK (data not shown), G6Pase, and PDK4 in WT cells, this effect was not decreased in null cells, suggesting that FKBP52 may be a negative regulator of AR at these genes. Like glucocorticoids (41), androgens are also known to up-regulate expression of CEACAM1 (37), and loss of FKBP52 was found to decrease this ability. Last, R1881 had no effect on expression of FAS.

Expression profile of GR, FKBP52, and related TPR proteins in metabolic tissues

To extrapolate these results to physiology, mice with targeted ablation of FKBP52 were examined. Western blot analysis of WT male mice (Fig. 2, A and B) revealed several GR bands ranging from 90 to 97 kDa in tissues that are metabolic targets of GR action [liver, soleus muscle, pancreas, white adipose tissue (WAT), and adrenals]. These bands are likely the multiple GR isoforms known to arise from alternative transcriptional splicing and translation

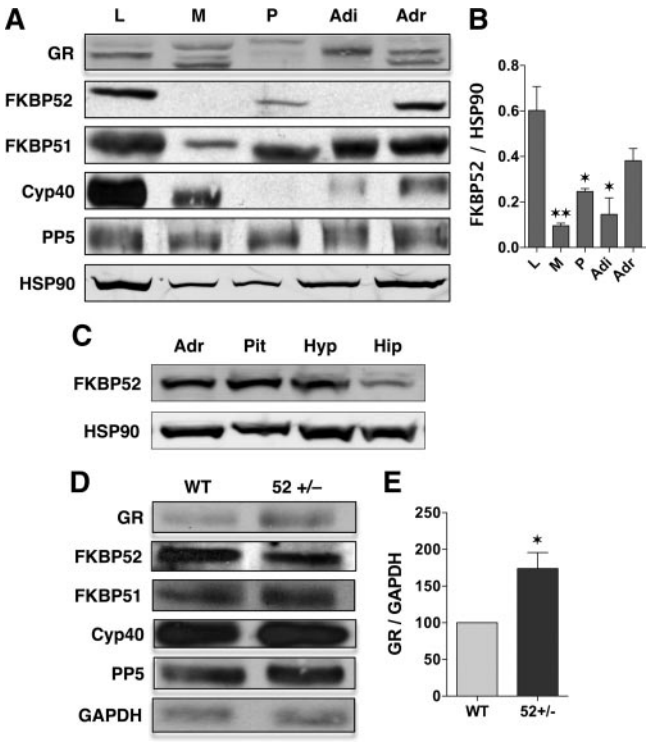

FIG. 2. Expression profile of GR and TPR proteins in metabolic and HPA axis tissues. A, Lysates from liver (L), soleus muscle (M), pancreas (P), white adipose (Adi), and adrenals (Adr) of WT mice were subjected to Western blotting using antibodies against indicated proteins. Antibody to Hsp90 was used as loading control. Quantitation of FKBP52 is shown in B. C, Western blot analysis of FKBP52 in adrenal (Adr), pituitary (Pit), hypothalamus (Hyp), and hippocampus (Hip). D, Western blot analysis of GR and TPR proteins in liver lysates from WT and FKBP52$^{+/-}$ animals maintained on RD. E, Quantitation of GR levels from B. Values represent means ± SEM. All results are representative of three independent experiments. Panel B: *, $P < 0.05$; **, $P < 0.01$ (*vs.* L). Panel E: *, $P < 0.05$ (*vs.* WT).

initiation sites (42). FKBP51 and PP5 were detected in all tissues tested, albeit at different levels of expression. Cyp40 was found in all tissues, except pancreas. Interestingly, expression of FKBP52 showed a tissue-specific pattern, with high-level expression in liver, pancreas, and adrenals, with little to no expression in muscle or adipose. This result identified the liver as the primary organ for potential alterations to metabolism in FKBP52-deficient mice. Because of the potential role of FKBP52 to affect endocrine control of adrenal GC secretion, expression of the protein was also assessed in hypothalamus, pituitary, and hippocampus (Fig. 2C). Except for hippocampus, in which expression was low, hypothalamus and pituitary showed levels of FKBP52 comparable with adrenals.

Because liver is an important organ for GR control of metabolism, levels of GR and TPR proteins in WT and FKBP52 mutant livers were measured by Western blot analysis (Fig. 2D). FKBP52$^{+/-}$ mice showed the expected half-gene dosage for expression of FKBP52. Expression levels of FKBP51, Cyp40, and PP5 were comparable between WT and mutant samples. As in MEF cells (Fig. 1), GR protein levels were higher in mutant liver samples compared with WT (Fig. 2D).

Normal obesity in FKBP52-deficient mice fed HF

To establish a link between FKBP52 deficiency and GR regulation of metabolism, we exposed WT and FKBP52 mutant mice to a diet rich in fat (45%). This diet is known to produce abnormalities characteristic of metabolic syndrome, such as visceral adiposity, weight gain, hyperglycemia, and insulin resistance in both human and murine models (43). Based on our molecular studies and the fact that GR is required for hyperglycemia in animal models of type 1 diabetes (44), we predicted that reduced GR activity in FKBP52 mutant mice would be protective against the adverse effects of HF feeding. Interestingly, the phenotypes obtained on the HF diet did not meet this expectation. After 4 wk of HF diet, FKBP52 mutant mice gained similar weight and showed the same amount of visceral adiposity as WT littermates (Fig. 3, A and B). Food intake during the HF diet was comparable between the two genotypes (Fig. 3C). In WT animals exposed to HF, TG levels showed either no increase or a nonstatistical increase (Fig. 3D). FFA levels, as expected, were significantly elevated in WT mice fed HF (Fig. 3E). In FKBP52 mutants fed HF, TG and FFA levels were comparable with WT.

Glucocorticoid resistance and hyperinsulinemia in FKBP52-deficient mice fed HF

In WT animals, the 4-wk HF diet showed a trend toward elevation in random-fed blood glucose that was not statistically significant compared with RD. However, a significant rise in blood glucose was seen in FKBP52$^{+/-}$ mice fed HF compared with mutant RD-fed controls. Glucose levels were also significantly different when comparing FKBP52$^{+/-}$ HF animals with WT HF counterparts (Fig. 4A). A similar pattern was seen with respect to serum insulin (Fig. 4B). As expected, HF caused a rise in insulin levels in WT animals, suggesting the onset of diet-induced insulin resistance in these mice. In FKBP52$^{+/-}$ animals, HF caused a more marked increase in insulin levels compared with WT mice, suggesting an increased propensity to develop insulin resistance in the mutant animals. Interestingly, corticosterone levels were elevated in mutant mice fed HF (Fig. 4C). Although it is tempting to speculate that the underlying cause of hyperglycemia is an elevated GC response in FKBP52$^{+/-}$ mice, our data suggest glucocorticoid resistance at target tissues as the better operating model (see below).

Hyperinsulinemia can result from increased insulin secretion at pancreatic β-cells and/or from decreased insulin clearance in the liver. Using C-peptide levels as an indica-

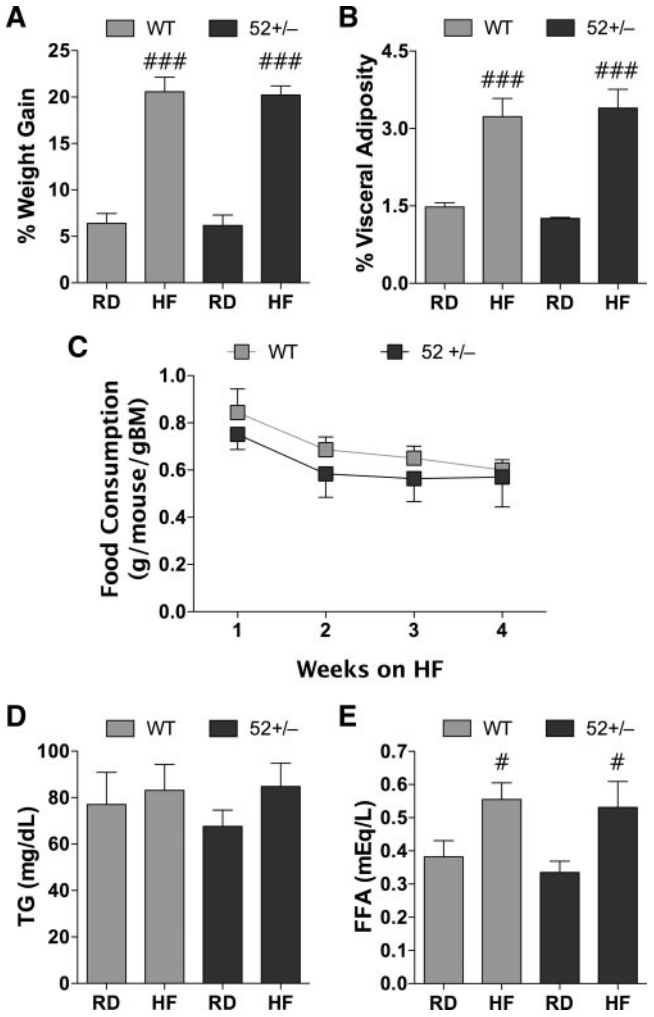

FIG. 3. Normal weight gain and serum lipid levels in FKBP52$^{+/-}$ animals fed an HF diet. Two-month-old WT and FKBP52$^{+/-}$ male mice were fed a RD or HF diet for 1 month, followed by measurement of percentage weight gain (A; n = 5–11), intra-abdominal (visceral) adipose tissue (percent of total body weight) (B; n = 9–16), HF food consumption normalized to body weight in grams (C; n = 8), serum TG (D), and serum FFAs (E; n = 8–17). All values represent means ± SEM. #, $P < 0.05$; ###, $P < 0.001$ (*vs.* RD).

tor of insulin secretion, we observed a significant increase in secretion in WT animals and a trend toward increased secretion in FKBP52 mutants in response to HF (Fig. 4D). HF did not significantly affect insulin clearance, measured by steady-state C-peptide/insulin molar ratio, in WT mice (Fig. 4E). In contrast, insulin clearance was greatly reduced in FKBP52 mutants fed HF. These results demonstrate that HF-induced hyperinsulinemia in FKBP52 mutants is most likely a combination of increased insulin secretion and reduced insulin clearance.

Because hepatic insulin clearance is principally mediated by CEACAM1 (34), we measured its mRNA level in liver by Northern blotting (data not shown) and quantitative RT-PCR (Fig. 4F). CEACAM1 levels were greatly reduced in HF-fed FKBP52 mutants compared with HF-fed WT mice. It was shown previously that CEACAM1 expression can be up-regulated by GCs (37), and here we showed that FKBP52 is required for that effect (Fig. 1D). We therefore speculate that reduction of CEACAM1 expression in the liver of FKBP52 mutants may be attributable to abrogated GR activity. Interestingly, CEACAM1 levels were also reduced in mutant mice fed an RD and in FKBP52$^{-/-}$ MEF cells not treated with Dex. This suggests a contribution by FKBP52 to basal CEACAM1 expression both *in vivo* and *in vitro*. However, CEACAM1 levels were not sufficiently reduced under RD conditions to cause significant increases in serum insulin or glucose (Fig. 4).

FKBP52 deficiency promotes hepatic steatosis

HF diet causes lipid accumulation in the liver, contributing to insulin resistance in both humans and mice. Consistently, HF induces lipid accumulation in WT livers, as assessed by Oil Red O staining (Fig. 5A). FKBP52 mutants on HF diet showed a larger number of macrovesicular lipid vacuoles, as well as vacant, nonstained areas, which are likely interstitial depots of fat washed away before staining. These observations were corroborated by biochemical measurement of hepatic TG content (Fig. 5B). Consistent with Oil Red O staining, FKBP52 mutants on HF diet showed a significant increase in hepatic TG content compared with HF-fed WT mice. Under RD conditions, FKBP52 mutant mice had a greater number of small lipid droplets, but this difference did not reach statistical significance.

Because lipid accumulation in FKBP52 mutants fed an HF diet was elevated, we next measured the level of proteins involved in *de novo* lipid synthesis (FAS) or secretion (MTP1 and ApoB100) by quantitative Western blotting. The results revealed no differences for MTP1 and ApoB100 between WT and FKBP52 mutants under RD and HF-diet conditions, suggesting that lipid secretion is not affected by the loss of FKBP52 (Fig. 5, C and D). In contrast, FAS was found to be elevated in the livers of mutant mice (Fig. 5E). FAS is an insulin-responsive gene that is partly responsible for the lipogenic effect of the hormone (45). Together, the data showed that FKBP52 mutant mice fed HF exhibited fatty livers and elevated insulin that correlated with hyperglycemia and reduced insulin clearance.

Reduced expression of hepatic gluconeogenic enzymes and elevated steatotic markers in FKBP52-deficient mice

Because FKBP52 mutants showed increased serum glucose levels under HF-diet conditions, we tested for altered expression of hepatic gluconeogenic enzymes by Northern blotting (data not shown) and by quantitative RT-PCR. Figure 6 shows that mRNA levels of PEPCK (data not shown),

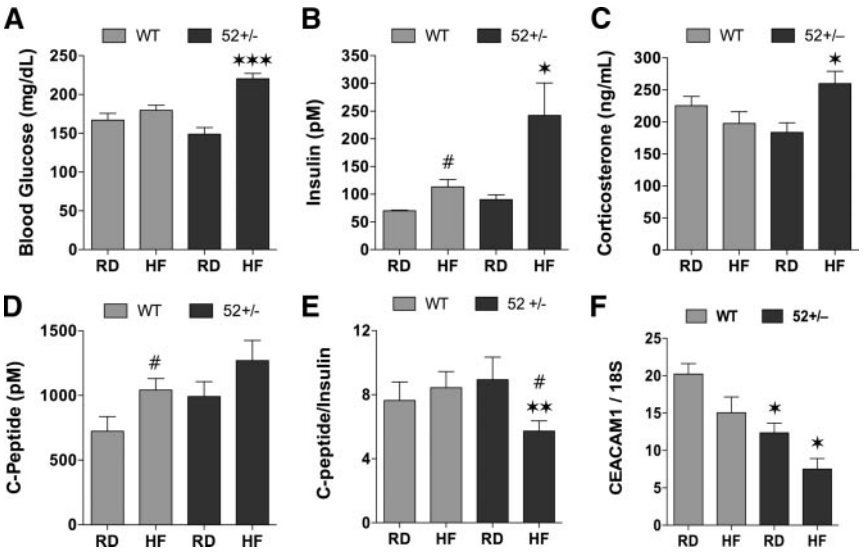

FIG. 4. Elevated corticosterone, glucose, and insulin and reduced insulin clearance and hepatic CEACAM1 expression in FKBP52$^{+/-}$ mice fed HF. Blood samples were obtained from WT and FKBP52$^{+/-}$ male mice fed RD and HF after 1 month of treatment to measure glucose levels (A), plasma insulin (B), corticosterone (C), and C-peptide (D). Insulin clearance was measured by calculating the C-peptide/insulin ratio (E). CEACAM1 levels in liver were measured by real-time PCR (F). Values represent means ± SEM (n = 4–23). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* WT). #, $P < 0.05$ (*vs.* RD).

PDK4, and G6Pase are significantly increased in WT animals fed HF. This observation is consistent with *in vivo* up-regulation of PDK4 in response to diets in which lipids, especially FFA, are the primary source of calories (46). In contrast, FKBP52-deficient mice showed significantly reduced expression of all three genes under HF-diet conditions. Because GR activity at these genes is clearly reduced in FKBP52-deficient MEF cells (Fig. 1), we speculate that the underlying cause of reduced enzyme expression in the steatotic livers is reduced GR activity. To assess the effect of FKBP52 loss on key markers of the steatotic state, measurements of peroxisome proliferator-activated receptor γ (PPARγ), FAS, sterol regulatory element-binding protein 1c (SREBP1c), and TNFα were also performed. The results show statistically elevated levels of all four markers in FKBP52 mutants fed HF.

Because little to no expression of FKBP52 was found in WAT and muscle (Fig. 2), it was expected that these organs would not have an intrinsic alteration of GR activity. This was tested by measurement of key GR-regulated genes in each tissue (Fig. 6). In skeletal muscle, expression of PDK4, glucocorticoid-inducible leucine zipper protein, and serum- and glucocorticoid-inducible kinase are up-regulated in response to GCs (47, 48). Our results in soleus muscle show that mRNA levels of each protein are either unchanged or somewhat elevated in FKBP52$^{+/-}$ mice fed HF compared with WT littermates. It is interesting to note that, similar to liver, HF diet increased expression of PDK4 in muscle, yet its expression was not reduced in the FKBP52 mutant mice. In WAT, levels of the GC-inducible enzyme hormone-sensitive lipase were the same when comparing mutant and WT mice. It can also be seen that levels of PPARγ and a key marker of adipocyte differentiation, adipocyte-specific protein 2 (49), were up-regulated by HF to the same degree in the mutant and WT animals. The latter facts are consistent with the equal visceral adipose weights obtained for each genotype (Fig. 3).

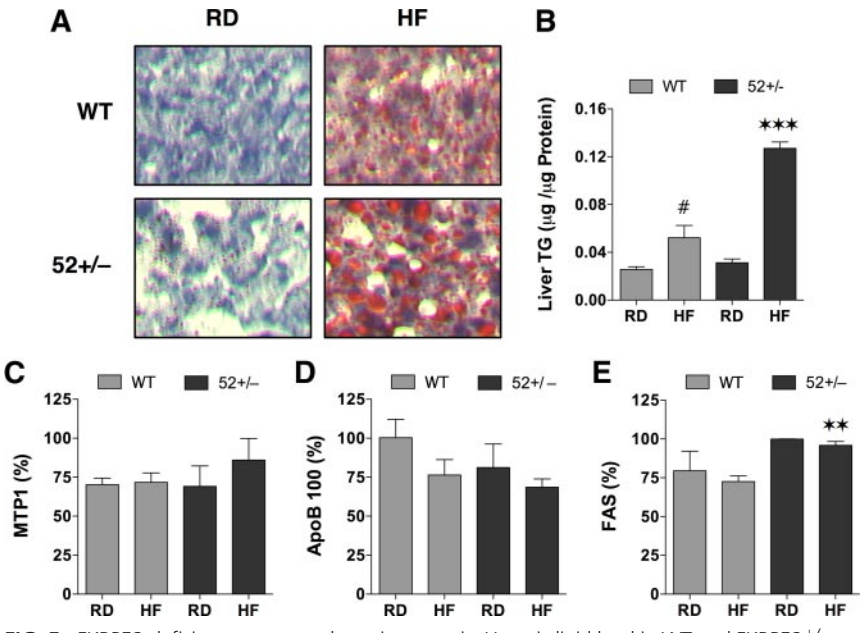

FIG. 5. FKBP52 deficiency promotes hepatic steatosis. Hepatic lipid load in WT and FKBP52$^{+/-}$ male mice fed RD and HF diets was measured by Oil Red O staining (A) and direct measurement of TG content (B; n = 3–5). C, Quantitative Western blot analysis of proteins important to lipid homeostasis (MTP1, ApoB100, and FAS) in liver. Densitometric values for each protein were normalized to GAPDH and expressed as percentage of the maximum value obtained after scanning (n = 4). All values represent means ± SEM. **, $P < 0.01$; ***, $P < 0.001$ (*vs.* WT). #, $P < 0.05$ (*vs.* RD).

Discussion

These studies represent the pioneer analysis of a steroid receptor cochaperone in metabolic processes *in vivo*. To achieve this, mice heterozygous for FKBP52 ablation were subjected to a 4-wk, HF-diet regimen known to induce a mild state of insulin resistance and other metabolic disturbances. On

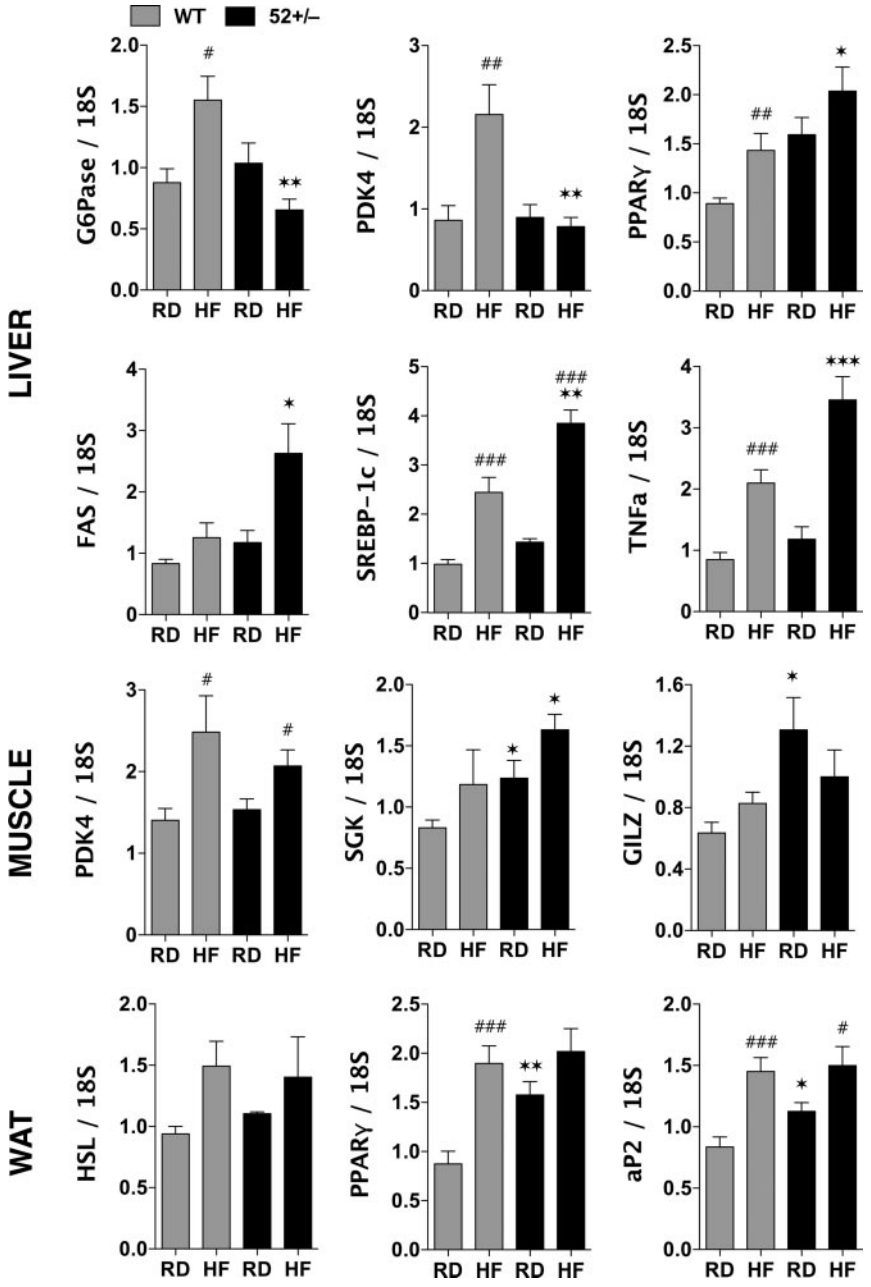

FIG. 6. Reduced expression of hepatic gluconeogenic enzymes and elevated markers of steatosis in FKBP52$^{+/-}$ mice fed HF. Expression of the indicated genes in liver, soleus muscle, and WAT from WT and FKBP52$^{+/-}$ male mice fed RD and HF diets was measured by real-time PCR (n = 4–8). Values represent means ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* WT). #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (*vs.* RD). *aP2*, Adipocyte-specific protein 2; GILZ, glucocorticoid-inducible leucine zipper; HSL, hormone-sensitive lipase; SGK, serum- and glucocorticoid-inducible kinase.

this diet, FKBP52-deficient mice showed an increased susceptibility to hyperglycemia and hyperinsulinemia that correlated with reduced insulin clearance and hepatic steatosis. Elevated serum corticosterone and hepatic GR levels were observed, yet the liver showed reduced expression of gluconeogenic genes, suggesting a state of glucocorticoid resistance. Based on these characteristics and because FKBP52 is strongly expressed in liver but nearly absent in muscle and adipose tissue, we propose that FKBP52-deficient animals are susceptible to diet-induced insulin resistance and hepatic steatosis, with the underlying biochemical defect being reduced hepatic gluconeogenesis.

The potential mechanisms by which reduced gluconeogenesis leads to hepatic lipid accumulation under HF-diet conditions are illustrated in the model of Fig. 7. Glucocorticoids control three important aspects of liver metabolism: gluconeogenesis, lipogenesis, and fatty acid oxidation (for review, see Ref. 1). In gluconeogenesis, several key enzymes are transcriptionally regulated by GR, including PEPCK and G6Pase, which control rate-limiting steps. In addition, PDK4 is an important GR-controlled enzyme that acts as a serine kinase to inactivate the pyruvate dehydrogenase complex (PDC). PDC catalyzes the conversion of pyruvate to acetyl-CoA for lipid synthesis when pyruvate is abundant in the cells (46, 50). Thus, PDK4 serves to conserve three carbon compounds (pyruvate, lactate, and glycerol) for gluconeogenesis by acting as a fuel selector switch. We propose that reduced expression of gluconeogenic enzymes in FKBP52 mutant mice fed HF causes a shunting of pyruvate to acetyl-CoA and *de novo* lipogenesis. Low expression of PDK4 further contributes to this effect. The source of pyruvate can either be glycolysis or glycerol, which is an abundant component of HF diets and which is converted in liver to GAPDH, an intermediate product in glucose synthesis/oxidation (16, 51, 52).

In response to HF, our WT animals showed moderate increases in serum insulin, hepatic lipid, and gluconeogenic enzymes, with little or no hyperglycemia. In contrast, FKBP52 mice fed HF did develop hyperglycemia, which was in keeping with their higher levels of insulin and hepatic lipid. This indicates that our 4-wk HF diet regimen was a moderate effector of insulin resistance in these animals and that loss of FKBP52 accelerates this response. However, it is interesting that hyperglycemia developed despite reduced gluconeogenic enzyme expression in the mutant mice. The most likely explanation for this is secondary insulin resistance in peripheral organs, such as skeletal

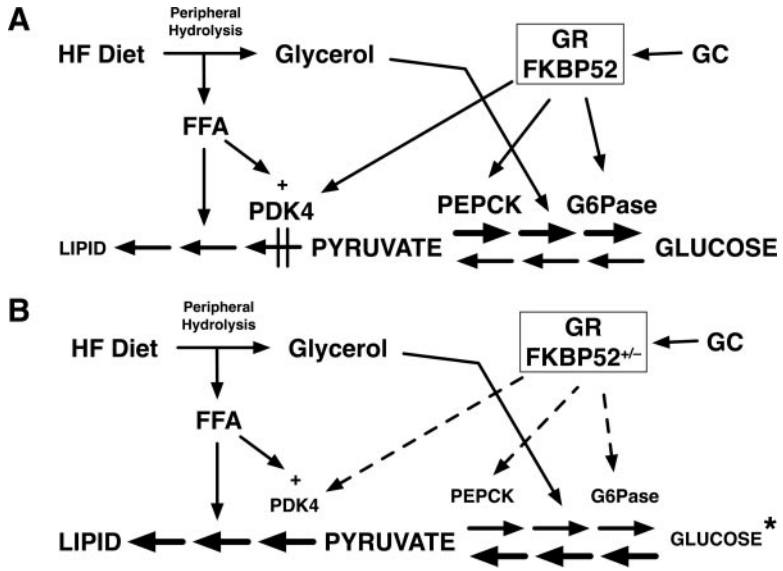

FIG. 7. The carbon shunting model of hepatic steatosis in FKBP52-deficient mice. Under HF-diet conditions, an excess of glycerol and FFAs are generated by peripheral hydrolysis. In WT mice (A), the excess glycerol enters the gluconeogenesis pathway as GAPDH through the initial actions of glycerol kinase (data not shown). FFAs simultaneously promote glucose production by increasing the expression and activity of PDK4, which along with PEPCK and G6Pase are also up-regulated by GC activation of GR. In FKBP52-deficient mice subjected to HF (B), reduced GR activity at PEPCK and G6Pase leads to reduced utilization of the glycerol-derived carbons for glucose synthesis, whereas reduced GR activity at PDK4 causes derepression of the PDC (data not shown). Thus, FKBP52 loss under HF causes a shunting of three-carbon substrates to pyruvate, leading to elevated lipid synthesis and increased susceptibility to hepatic steatosis. Contributing to the steatotic phenotype is stimulation of lipogenesis by hyperinsulinemia (data not shown). *, Although a reduction of intrinsic hepatic glucose production is proposed in these mice, serum hyperglycemia is observed that may result from secondary peripheral insulin resistance (untested).

muscle and adipose tissue, especially because FKBP52 appears to be missing or greatly reduced in these organs and can therefore make no direct contribution to peripheral insulin resistance in the mutant mice. Such an outcome would be consistent with other examples of hyperglycemia caused by secondary insulin resistance in muscle arising from hyperinsulinemia attributable to a primary defect in liver (33, 53). In such cases, hepatic TG can be redistributed to muscle in response to elevated insulin secretion, leading to impairment of glucose uptake (33, 53). It is also possible that hypercorticosteronemia is contributing to peripheral insulin resistance, because GR activity in muscle and adipose appears to be normal in the FKBP52 mutants and because GCs are well-known antagonists of insulin action in muscle. Indeed, levels of two GR markers in soleus muscle were elevated in mutant mice fed HF, suggesting overstimulation by corticosterone. To properly investigate these hypotheses, follow-up studies will be necessary that measure a variety of GR and insulin resistance markers and that use metabolic flux assays, including glucose tolerance tests and hyperinsulinemic-euglycemic clamps.

Although less well-documented than gluconeogenesis, GCs are also known to promote hepatic lipogenesis, both *in vivo* and in isolated hepatocytes, through a direct stimulation of lipogenesis and by inhibition of fatty acid oxidation (23, 54, 55). Indeed, in mice with liver-specific ablation of GR, reduced accumulation of lipid has been noted (44, 56). In support of this lipogenic model is the result here that Dex increases FAS gene expression in WT MEF cells and that loss of FKBP52 may attenuate this response. This suggests that a global reduction of hepatic GR activity through FKBP52 deficiency should lead to reduced lipid content rather than the elevated fatty liver state we have observed. The most likely explanation for this paradox is that hyperinsulinemia compensates for reduced GR activity at lipogenic genes. For example, we observed not only elevated levels of hepatic FAS in mutant mice fed HF but also increased expression of SREBP1c, a transcriptional regulator of FAS gene expression that is induced by insulin (57, 58).

We have ascribed the diet-induced metabolic perturbations seen in FKBP52 mutant mice to reduced GR activity. The overriding reason for this is reduced expression of four GR target genes (CEACAM1, PDK4, PEPCK, and G6Pase) in mutant mice fed HF. Because these genes were also reduced in FKBP52-deficient MEF cells treated with Dex, it is very likely that GR is the mediator of the FKBP52 effect under HF conditions, yet potential contributions by other members of the nuclear receptor family controlled by FKBP52 must be considered. Because FKBP52 requires Hsp90 as a docking site within receptor complexes (59–61), we can categorically exclude members of the type II nuclear receptors, such as thyroid and retinoic acid receptors because these do not form complexes with Hsp90 (59, 62). However, PRs, ARs, and estrogen receptors (ERs) do interact with Hsp90 (5). Moreover, AR and ER are known to control important metabolic pathways, perturbations of which are linked to diabetes and insulin resistance (63), yet ER is not a likely candidate because *in vivo* and *in vitro* studies have shown that FKBP52 is not an important regulator of ER signaling (10, 64) and because estradiol causes down-regulation of hepatic gluconeogenic enzymes (65). FKBP52 is essential for PR activity in the uterus, resulting in female sterility (9, 10). However, $FKBP52^{+/-}$ females have normal fertility, showing that half-gene dosage for FKBP52 may not affect PR. Because the present study used $FKBP52^{+/-}$ male mice and because no effect of progesterone on hepatic glu-

coneogenic enzymes has been described, it is not likely that PR is a contributor to our findings. A study in mice with liver-specific ablation of AR showed that HF feeding caused hepatic lipid accumulation that correlated with elevated expression of gluconeogenic enzymes (40). Thus, although reduced AR activity contributes to steatosis, it appears to do so by a distinct mechanism that promotes rather than inhibits gluconeogenesis. Moreover, our analysis of AR in MEF cells suggests that loss of FKBP52 is neutral or increases AR activity at gluconeogenic genes. However, AR may be contributing to part of our phenotype, because it too up-regulates CEACAM1 and that activity is dependent on FKBP52.

In mutant mice, elevated serum corticosterone and hepatic GR content were observed. This observation is consistent with reduced GR activity and a state of glucocorticoid resistance (66, 67). To date, most examples of GC resistance arise from GR mutations that compromise hormone binding or transcriptional activity, leading to compensation by the hypothalamus-pituitary-adrenal (HPA) axis with elevated secretion of ACTH and GCs (67, 68). The resistance arising in the mutant FKBP52 mice is most likely tissue specific because of the restricted expression profile of FKBP52 in peripheral organs, yet FKBP52 was also found expressed in the hypothalamus, pituitary, and adrenals. Thus, elevated corticosterone may also result from impairment of feedback regulation at the HPA axis, as reported in the GR knockout mouse (69). Deciphering the relative contribution of peripheral *vs.* HPA contribution to our phenotype will require analyses of intrinsic endocrine functions.

Taken as a whole, this study underscores the need for GR activity in the liver under metabolic stress, such as the HF diets. As discussed above, this most likely involves conservation of glycerol and other three-carbon sources for the production of glucose in the presence of excess FFAs. Paradoxically, this would suggest that GC activation of hepatic GR is actually beneficial in response to the typical HF diet. In most studies, GC stimulation has been seen as deleterious, promoting lipid buildup and necessary for the hyperglycemic response in diabetes. However, under real-world diets and stresses, these deleterious actions most likely arise from chronic, elevated secretion of GCs. Based on our results, we propose that a normal, measured GC response is beneficial, helping to reduce hepatic lipid load in the face of excess dietary fat. As such, development of new agents that increase activity or expression of FKBP52 should prove useful in treating diet-induced metabolic disorders.

Acknowledgments

We are indebted to Dr. Jennifer Hill, Dr. Joseph Marino, Mats Fernstrom, Anthony DeAngelis, and Dr. Jill Gloeckler-Schroeder for help with animal dissections and to Dr. Cara M. Gatto-Weis for advice on hepatic lipid staining.

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This work was supported by National Institutes of Health Grants DK70127 (to E.R.S. and W.S.) and DK54254 (to S.M.N.) and by United States Department of Agriculture Grant 38903-02315 (to S.M.N). T.D.H. was supported by predoctoral National Institutes of Health National Research Service Award F31DK84958.

Disclosure Summary: The authors have nothing to disclose.

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