Gipr Is Essential for Adrenocortical Steroidogenesis; However, Corticosterone Deficiency Does Not Mediate the Favorable Metabolic Phenotype of *Gipr*$^{-/-}$ Mice

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Glucose-dependent insulinotropic polypeptide (GIP) promotes glucose-dependent insulin secretion. However, GIP also enhances glucocorticoid secretion and promotes adiposity. Because obesity and diabetes are glucocorticoid dependent, we examined whether the effects of GIP on energy balance and glycemia are regulated by glucocorticoids using pharmacological activation of GIP receptor (GIPR) signaling with [D-Ala^2]GIP in mice and in Y1 adrenocortical cells. Genetic elimination of GIPR activity was also studied in normal- and high fat (HF)-fed *Gipr* deficient (*Gipr*$^{-/-}$) mice. [D-Ala^2]GIP increased murine corticosterone levels in a GIPR-dependent manner. Conversely, basal corticosterone levels were reduced, whereas food deprivation resulted in significantly enhanced plasma corticosterone levels in *Gipr*$^{-/-}$ mice. [D-Ala^2]GIP increased cAMP levels, activated ERK1/2, increased expression of steroidogenic genes, and increased neutral lipid storage in Y1GIPR cells. *Gipr*$^{-/-}$ adrenal glands demonstrated a twofold upregulation of the ACTH receptor mRNA and increased sensitivity to ACTH ex vivo. Although HF-fed *Gipr*$^{-/-}$ mice exhibited significantly lower plasma corticosterone, glucocorticoid-treated HF-fed *Gipr*$^{-/-}$ mice had similar energy balance and glycemia compared with *Gipr*$^{+/+}$ controls. Hence, although the *Gipr* is essential for adrenal steroidogenesis and links HF feeding to increased levels of corticosterone, reduced glucocorticoid levels do not significantly contribute to the enhanced metabolic phenotypes in HF-fed *Gipr*$^{-/-}$ mice.

Glucose-dependent insulinotropic polypeptide (GIP) is a 42–amino acid peptide hormone released from intestinal K cells upon nutrient ingestion (1). The original description of GIP bioactivity encompassing inhibition of gastric emptying was subsequently expanded to include potentiation of glucose-dependent insulin secretion. GIP also can stimulate lipogenesis (2,3) and increase the activity of lipoprotein lipase in adipocytes in part through stimulatory effects on resistin secretion (4,5), thus enhancing fatty acid incorporation into adipose tissue.

Complementary insights into the physiological importance of endogenous GIP action have been obtained from studies using antagonists, elimination of GIP-producing K cells, or receptor knockout mice. Reduction or elimination of GIP action promotes resistance to genetic and high fat (HF) diet–induced obesity, increased energy expenditure and activity, reduced fat deposition in nonadipose and adipose depots, improved insulin sensitivity, and lower plasma resistin levels (5–9). Therefore, it remains unclear whether enhancement or reduction of GIP action is the preferred therapeutic strategy for the treatment of obesity-associated type 2 diabetes.

An additional extrapancreatic locus of GIP action is the adrenal cortex. Expression of the GIP receptor (GIPR) has been demonstrated in the adrenal cortex by radioligand binding (10) and RT-PCR (11) in rodents; however, the GIPR is not expressed in normal human adrenal glands (12). Ectopic expression of the GIPR in the human adrenal gland causes significant hypercortisolemia after meal ingestion and leads to Cushings syndrome (13), demonstrating that human GIPR activation is capable of robustly activating adrenal glucocorticoid secretion. Indeed, GIP administration increases corticosterone levels in rats (10), and isolated rat adrenocortical zona fasciculata/reticularis cells respond to GIP in a cAMP-dependent protein kinase manner (10). However, the mechanisms through which GIP stimulates glucocorticoid secretion and the physiological importance of adrenal GIP action remains poorly understood.

Chronic elevations of glucocorticoid levels promote obesity and/or metabolic syndrome, and many rodent models of obesity or insulin resistance, including *ob/ob* and *db/db* mice, Zucker and ZDF rats, HF-fed rodents, and streptozotocin-induced diabetic rodents, exhibit elevations in glucocorticoids (14–17). Furthermore, adrenalectomy or glucocorticoid receptor (GR) antagonists ameliorate or in some cases prevent diabetes and obesity phenotypes (18,19). Accordingly, we hypothesized that antagonism or removal of the GIPR-dependent stimulation of corticosterone secretion after nutrient ingestion may contribute to one or more of the phenotypes exhibited by *Gipr*$^{-/-}$ mice. We now show that activation or disruption of the GIPR modulates glucocorticoid levels in mice, and GIP directly enhances glucocorticoid synthesis and secretion in adrenocortical cells. However, the reduction in glucocorticoid levels observed in *Gipr*$^{-/-}$ mice does not substantially contribute to the phenotypes of improved glucose tolerance and resistance to diet-induced obesity in HF-fed *Gipr*$^{-/-}$ mice.

RESEARCH DESIGN AND METHODS

All experiments were done using male mice acclimatized to handling before commencement of experiments. Wild-type C57BL/6 mice were obtained from the Toronto Centre for Phenogenomics (TCP; Toronto, ON, Canada). The generation and characterization of *Gipr*$^{-/-}$ mice has been described previously (20). Mice were housed under a 12-h light/dark cycle in the TCP animal

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facility with free access to standard rodent diet (2018, 18% kcal from fat; Harlan Teklad, Mississauga, ON, Canada) and water, except where otherwise noted. All procedures were conducted according to protocols and guidelines approved by the TCP Animal Care Committee.

Acute stimulation of corticosterone by [D-Ala^2]GIP. Overnight fasted C57BL/6 mice or ad libitum fed *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice were injected intraperitoneally with 24 nmol/kg [D-Ala^2]GIP (California Peptide Research Inc., Napa, CA) or saline. Blood for corticosterone was obtained from the tail vein.

Ex vivo ACTH sensitivity. Adrenal glands from *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice were obtained after 5 weeks of HF diet, and responsiveness to ACTH was measured as described (21). Briefly, adrenals were excised, cleaned, halved, and incubated in vitro in Dulbecco's modified Eagle's medium with 25 mmol/L HEPES and 0.1% w/v BSA. After preincubation for 120 min, the medium was changed and adrenals were incubated for 60 min to determine basal corticosterone production. Medium was changed again and 1 nmol/L ACTH (Sigma-Aldrich, St. Louis, MO) was applied for 60 min. Medium was frozen for analysis of corticosterone levels.

Basal corticosterone measurements. Basal blood samples were obtained at the diurnal peak (1800 h) and trough (0900 h) of the corticosterone rhythm in *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice. Mice were in a quiet room for at least 1 h prior to sampling, and blood for corticosterone was obtained from the tail vein within 1 min of opening the cage.

Stress experiments. *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice were exposed to various stress regimens, including 24-h food deprivation, 30-min restraint stress, ACTH stimulation test (5 units/kg), and insulin-induced hypoglycemia (1.0 units/kg Humulin; Lilly, Toronto, ON, Canada), with at least 2 weeks between experiments for recovery. For dietary stress, mice were fed a HF diet with 45% fat (Research Diets, New Brunswick, NJ) for 2 weeks after which blood was obtained by cardiac puncture after cervical dislocation. The adrenal glands, pituitary, and hypothalamic wedge were immediately dissected and stored in RNAlater (Qiagen, St. Louis, MO) until frozen at −80°C.

Y1 adrenocortical cells. Mouse Y1 adrenocortical cells were a gift from B. Schimmer (University of Toronto). Cells were cultured in Ham's F10 Media (Invitrogen, Burlington, ON, Canada) supplemented with 15% heat-inactivated horse serum, 2.5% heat-inactivated FBS, and penicillin/streptomycin. The rat GIPR cDNA (22) was transfected into Y1 cells using lipofectamine 2,000 transfection reagent (Invitrogen, Burlington, ON, Canada). After selecting for cells stably expressing the vector through incubation in 100 μg/mL G418 (Invitrogen, Burlington, ON, Canada), cells were maintained as a pooled population of mixed G418-resistant clones (Y1GIPR cells). Cells for experiments were plated in Primaria-coated six-well culture plates (BD Falcon, Mississauga, ON, Canada) and treated with human GIP (California Peptide Research Inc.), [D-Ala^2]GIP (California Peptide Research Inc.), 10 μmol/L Forskolin (Sigma-Aldrich), or 1 nmol/L ACTH. cAMP production was measured as described (22) after serum starvation for 24 h and assayed by radioimmunoassay (Biomedical Technologies, Stoughton, MA). Progesterone secretion was measured in cells incubated in complete media for 24 h and assayed by enzyme immunosorbent assay (Cayman Chemical Company, Ann Arbor, MI). Neutral lipid content of Y1 cells was quantified by elution of Oil Red O in isopropanol and measurement of the absorbance of the eluant at 492 nm (23)

Immunoblotting of Y1 cells. Cells were grown to ~70% confluence in six-well culture plates. Prior to the experiment, cells were serum starved in α–minimum essential medium (Invitrogen, Burlington, ON, Canada) for 72 h. Cells were lysed and immunoblotted as previously described (24). Membranes were immunoblotted for phosphorylated extracellular signal–related kinase (ERK; 1:500) and total ERK (1:500, Cell Signaling Technologies, Danvers, MA) and for Hsp90 (1:3,500; BD Transduction Laboratories, Mississauga, ON, Canada), followed by the appropriate secondary antibodies.

Adrenal gland histology. Adrenal glands were isolated from *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice and cleaned of extraneous fat. Adrenals were fixed in 10% neutral buffered formalin for hematoxylin-eosin staining or frozen in Tissue-Tek (Optimal Cutting Temperature Compound; Sakura Finetek, Torrance, CA) for staining of neutral lipids with Oil Red O.

Corticosterone supplementation experiments. At aged 10–13 weeks, *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ littermates were fed ad libitum a HF diet, with 45% kcal from fat (Research Diets) for 22 weeks. Concurrently, mice were randomized to receive water supplemented with 5 μg/mL corticosterone (Sigma-Aldrich) dissolved in ethanol or vehicle (0.04% ethanol) for 22 weeks. This dose was chosen on the basis of preliminary experiments demonstrating that this dose increased plasma corticosterone levels but did not cause dark-phase hypercorticosteronemia or significantly suppress plasma ACTH levels. Assessment of fat and lean mass was done using a whole-body magnetic resonance analyzer (Echo Medical Systems, Houston, TX) after 16 weeks of treatment. For assessment of food and water intake after 14 weeks of treatment, mice were individually housed for the preceding week. Mice were fasted during the day (0800 to 1600 h), weighed, and then given preweighed rodent food. Food was reweighed at 0900 h (nocturnal food intake) and then at 0900 h 3 days later for ad libitum food intake. After 14 and 20 weeks of corticosterone supplementation, water intake was measured in individually housed mice over the course of 3 or 7 days, respectively. V_{O_2} and respiratory exchange ratio were determined after 15 weeks of treatment by indirect calorimetry using an Oxymax System (Columbus Instruments, Columbus, OH). V_{O_2} was measured at 15-min intervals between 1200 and 0700 h for 19 h after at least 2 h acclimatization to the metabolic chamber where mice had free access to food and the appropriately supplemented water. Locomotor activity was simultaneously measured as the number of independent beam breaks (ambulatory activity) in Opto M3 activity monitors (Columbus Instruments). After 16 and 17 weeks of treatment, an oral glucose tolerance test and intraperitoneal glucose tolerance test were performed (1.5 mg/g glucose), respectively, after an overnight 16-h fast. After 18 weeks of treatment, an insulin tolerance test (ITT) was performed (1.2 units/kg insulin, Humulin R, 100 units/mL; Lilly) after a 5-h fast. Blood was sampled from the tail vein, and blood glucose levels were measured using a Glucometer Elite blood glucose meter (Bayer, Toronto, ON, Canada). Basal plasma corticosterone samples were obtained from the tail vein of individually housed mice after 10 and 20 weeks of treatment at the diurnal peak (1900 h) and trough (0800 h) of the corticosterone rhythm. Mice were transported to the sampling room 3 h prior to the 1900 h sample to acclimatize and left in the room overnight for the 0800 h sample.

RNA isolation. Total RNA was extracted from adrenal glands, hypothalamus, adipose tissue, or pituitary using the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). RNA from Y1 cells was extracted using TriReagent (Molecular Research Center Inc., Cincinnati, OH).

Real-time quantitative RT-PCR. First-strand cDNA was synthesized from total RNA using the SuperScript II or III synthesis system (Invitrogen, Carlsbad, CA) and random hexamers. Real-time quantitative (q)RT-PCR was carried out with the ABI Prism 7900 Sequence Detection System using TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The relative mRNA transcript levels were quantified with the $2^{-\Delta\Delta Ct}$ method (25), using cyclo*phillin* or *gapdh* as internal control genes.

Hormone assays. Plasma was separated by centrifugation and stored at −80°C until assayed. Corticosterone and ACTH (ICN Biomedicals, Montreal, QC, Canada) and plasma insulin (ALPCO, Salem, NH) were assayed by radioimmunoassay.

Statistical analysis. All results are expressed as means ± SE. Statistical significance was assessed by ANOVA and where appropriate, a Student *t*-test using GraphPad Prism 4 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered to be statistically significant.

RESULTS

The GIPR agonist [D-Ala^2]GIP increases plasma corticosterone in mice via the GIPR. [D-Ala^2]GIP administration to overnight fasted C57BL/6 mice induced a brisk rise in corticosterone levels (Fig. 1*A*); in contrast, no increase in plasma corticosterone levels occurred after [D-Ala^2]GIP administration in *Gipr*$^{-/-}$ mice (Fig. 1*B*). To determine if chronic GIPR activation alters corticosterone levels, we assessed diabetic mice treated with saline or [D-Ala^2]GIP twice daily for 60 days (26). Corticosterone levels were more than threefold higher in mice chronically injected with [D-Ala^2]GIP (Fig. 1*C*).

Basal corticosterone levels are reduced in *Gipr*$^{-/-}$ mice. We next assessed plasma corticosterone in *Gipr*$^{-/-}$ mice. Corticosterone levels were reduced in *Gipr*$^{-/-}$ mice (Fig. 1*D*) during the peak of the circadian corticosterone rhythm at 1800 h, 1 h prior to their dark cycle. The lower corticosterone levels in *Gipr*$^{-/-}$ mice were not associated with simultaneous differences in glycemia (data not shown). We next examined the corticosterone response to stress in *Gipr*$^{-/-}$ mice. Plasma corticosterone responses to restraint stress or insulin-induced hypoglycemia were comparable across genotypes (Fig. 2). In a similar manner, exogenous administration of ACTH produced robust yet comparable increases in plasma corticosterone in *Gipr*$^{+/+}$ versus *Gipr*$^{-/-}$ mice (Fig. 2). In contrast, corticosterone levels were more than threefold higher in *Gipr*$^{-/-}$ mice after 24 h of food deprivation (Fig. 2). Glucose levels were

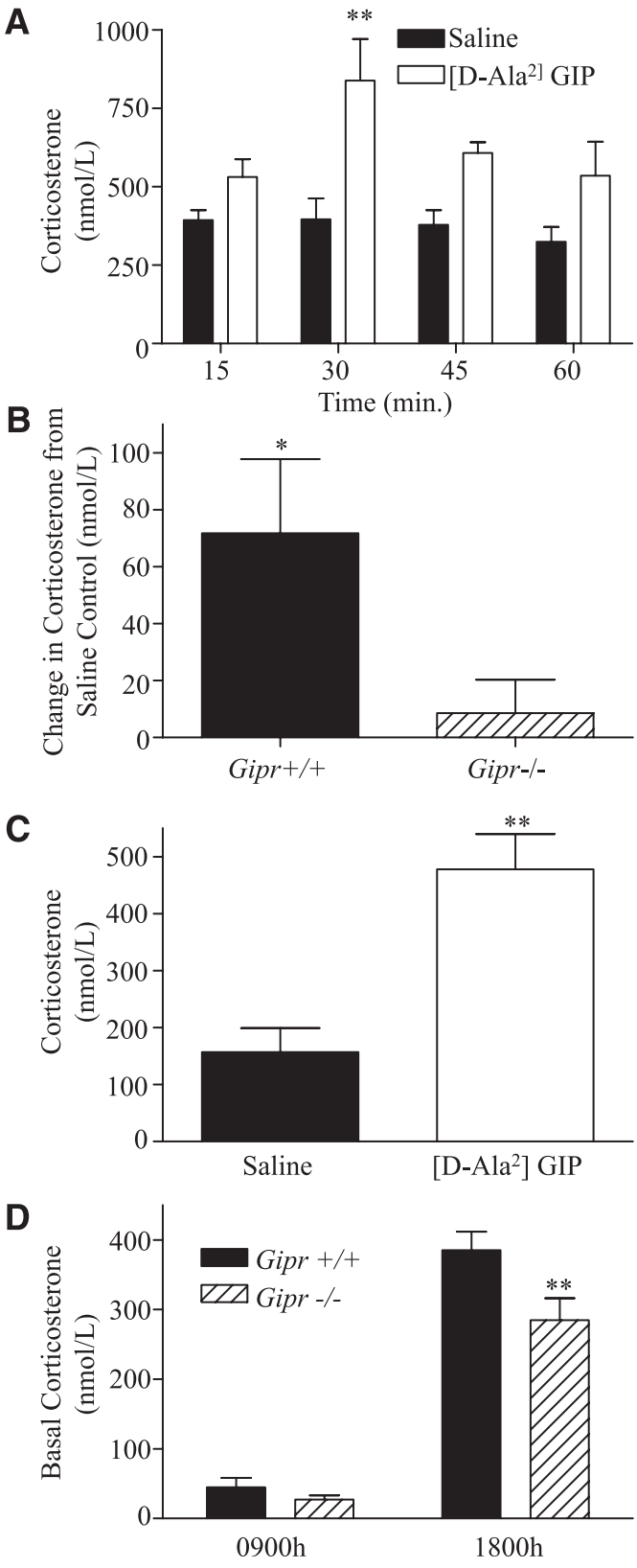

FIG. 1. GIPR signaling alters plasma corticosterone levels in mice. *A*: [D-Ala^2]GIP induces a rise in corticosterone levels. [D-Ala^2]GIP (24 nmol/kg) was injected intraperitoneally into overnight-fasted C57BL/6 mice and corticosterone sampled from the tail vein at 15, 30, 45, or 60 min after injection (n = 4–6 per group). *B*: Intraperitoneal injection of [D-Ala^2]GIP (24 nmol/kg) increases corticosterone in *Gipr*$^{+/+}$ but not *Gipr*$^{-/-}$ mice 30 min after injection (n = 10 per group). *C*: Twice-daily injection of [D-Ala^2]GIP (24 nmol/kg) for 60 days in streptozotocin-injected mice (26) increases corticosterone levels more than threefold (n = 8–12). *D*: Basal, unstressed corticosterone levels are reduced in *Gipr*$^{-/-}$ mice compared with wild-type littermates (n = 7–10). *P < 0.05, **P < 0.01 [D-Ala^2]GIP vs. saline-injected control or *Gipr*$^{-/-}$ vs. *Gipr*$^{+/+}$.

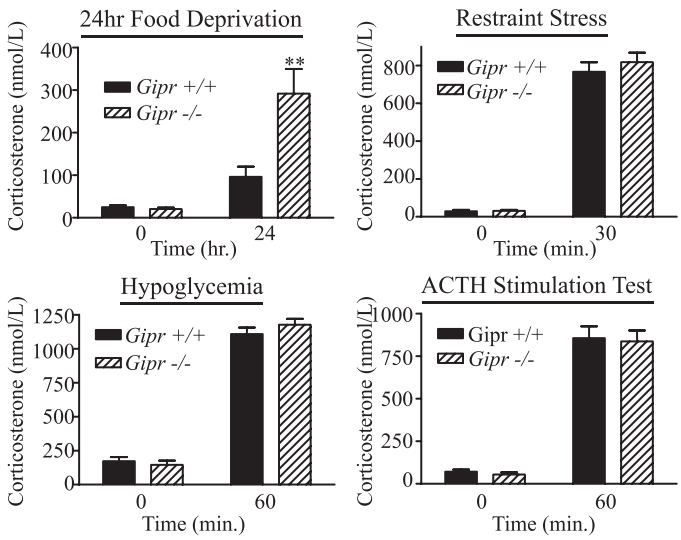

FIG. 2. Food deprivation stress selectively enhances corticosterone responses in *Gipr*$^{-/-}$ mice. *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice were exposed to various stress paradigms with at least 2 weeks between experiments, and corticosterone was measured in blood samples obtained from the tail vein. For food deprivation stress, blood samples were obtained in the morning between 0900 and 1100 h, after a 24 h fast. Mice were subjected to restraint stress by placement in a perforated 50-mL plastic conical tube for 30 min. For ACTH stimulation, ACTH (Sigma-Aldrich) was injected at a dose of 5 units/kg i.p. at 0900 h. For insulin-induced hypoglycemia, mice were fasted for 5 h and given 1.0 unit/kg insulin (Humulin R, 100 units/mL; Lilly) by intraperitoneal injection, and corticosterone samples were obtained between 1300 and 1600 h. **P < 0.01 vs. *Gipr*$^{+/+}$ mice (n = 6–14 per group).

not different between *Gipr*$^{+/+}$ versus *Gipr*$^{-/-}$ mice during these experiments (data not shown).

Corticosterone levels are reduced in HF-fed *Gipr*$^{-/-}$ mice in the absence of reduced hypothalamic–pituitary axis activation. HF feeding increases basal corticosterone levels (17). Accordingly, we examined whether the *Gipr* links excess nutrients to increased plasma corticosterone. Remarkably, basal corticosterone levels were reduced by >50% in *Gipr*$^{-/-}$ mice after 2 weeks of HF feeding (Fig. 3*A*). However, levels of plasma ACTH (Fig. 3*A*), pituitary proopiomelanocortin (*pomc*) (Fig. 3*B*), and hypothalamic corticotrophin-releasing hormone (*crh*) mRNA transcripts (Fig. 3*C*) were comparable in *Gipr*$^{+/+}$ versus *Gipr*$^{-/-}$ mice. GR expression also was similar in the pituitary, hypothalamus, and epididymal white adipose tissue of *Gipr*$^{+/+}$ versus *Gipr*$^{-/-}$ mice (Fig. 3*B–D*), and expression of the glucocorticoid synthesizing enzyme 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) was similar between genotypes (Fig. 3*D*). Hence, elimination of the *Gipr* is not associated with generalized perturbations in key molecular components of the hypothalamic–pituitary axis (HPA) despite expression of *Gipr* mRNA at all levels of the HPA axis (Supplementary Fig. 1), nor is it associated with alterations in several genes regulating glucocorticoid synthesis and action in key target tissues.

***Gipr*$^{-/-}$ mice exhibit altered adrenal expression of steroidogenic genes and increased sensitivity to ACTH.** To delineate mechanism(s) through which GIPR signaling controls corticosterone in mice, we assessed the expression of genes regulating adrenal steroidogenesis in *Gipr*$^{+/+}$

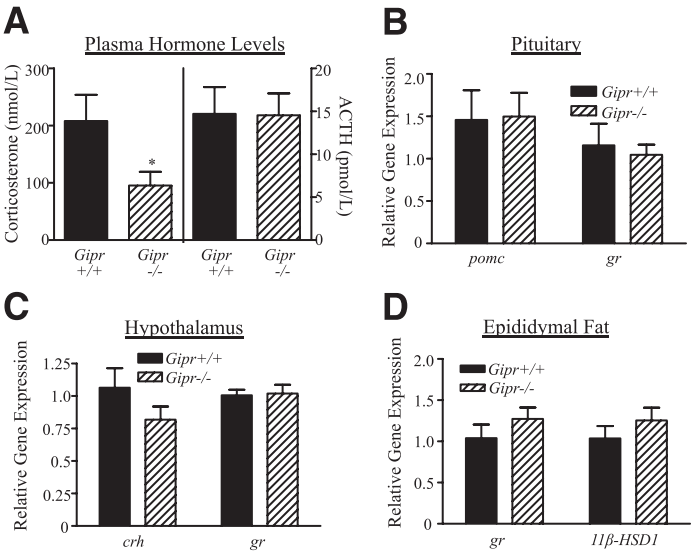

FIG. 3. Corticosterone levels are reduced in HF-fed *Gipr*$^{-/-}$ mice. *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice were fed a 45% HF diet for 2–3 weeks and basal, unstressed HPA parameters measured by real-time qRT-PCR. *A*: Plasma corticosterone levels were reduced in *Gipr*$^{-/-}$ mice without changes in plasma ACTH levels. Gene expression of *pomc* in the pituitary gland (*B*) and *crh* in the hypothalamus (*C*) were not altered in *Gipr*$^{-/-}$ mice. In a similar manner, GR expression was not changed in the pituitary (*B*), hypothalamus (*C*), or epididymal white adipose tissue (*D*) of *Gipr*$^{-/-}$ mice. Gene expression of the glucocorticoid synthesizing enzyme 11β-HSD1 (*D*) was not changed in *Gipr*$^{-/-}$ mice. **P* < 0.05 vs. *Gipr*$^{+/+}$ mice (*n* = 4–11 per group).

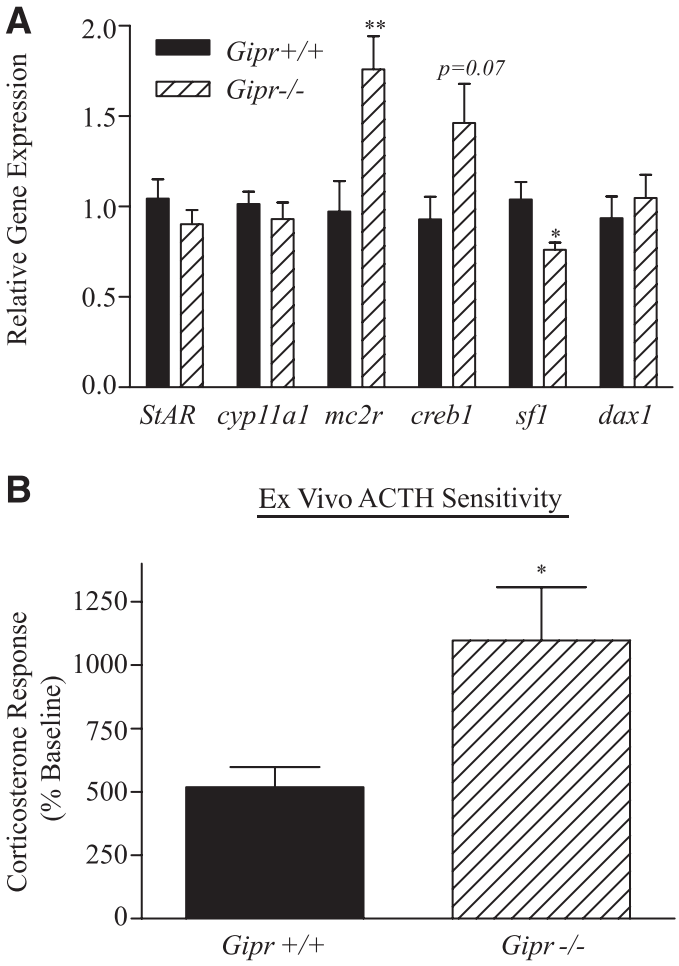

FIG. 4. Altered expression of steroidogenic genes and increased sensitivity to ACTH in adrenal glands of *Gipr*$^{-/-}$ mice. *A*: Expression of genes involved in adrenal steroidogenesis were measured by real-time qRT-PCR in adrenal glands from *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice after 2 weeks of HF feeding (*n* = 8–9 per group). *B*: Ex vivo adrenal sensitivity to ACTH (1 nmol/L) was measured in adrenal glands from *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice after 5 weeks HF feeding (*n* = 5 per group). **P* < 0.05, ** *P* < 0.01 vs. *Gipr*$^{+/+}$ mice.

versus *Gipr*$^{-/-}$ mice after 2 weeks of HF feeding. Levels of *StAR*, *cyp11A1*, and *dax1* mRNA transcripts were comparable in *Gipr*$^{-/-}$ mice, *sf1* mRNA transcripts were significantly reduced ($P < 0.05$), *creb1* mRNA transcripts were increased ($P = 0.07$), and *mc2r* mRNA transcripts were significantly higher ($P < 0.01$) in *Gipr*$^{-/-}$ adrenals (Fig. 4*A*). To assess whether increased levels of mRNA transcripts for the ACTH receptor (*mcr2*) reflected functionally increased sensitivity to ACTH, we administered a low dose of ACTH (1 nmol/L) to *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mouse adrenals ex vivo. A significantly greater corticosterone response to ACTH was detected from *Gipr*$^{-/-}$ mouse adrenals, consistent with enhanced ACTH sensitivity (Fig. 4*B*).

GIP regulates lipid accumulation in adrenocortical cells. Because GIP enhances adrenal corticosterone secretion, we hypothesized that the *Gipr* regulates expression of genes controlling adrenal cholesterol homeostasis. Although the majority of mRNA transcripts encoding genes within the cholesterol biosynthetic and transport pathways were not differentially expressed in *Gipr*$^{-/-}$ versus *Gipr*$^{+/+}$ adrenal glands (Fig. 5*A*), expression of *sr-b1* was significantly reduced in *Gipr*$^{-/-}$ adrenal glands (Fig. 5*A*). Since SR-B1 is responsible for selective uptake of cholesterol esters from HDL, the primary source of adrenal cholesterol stores in mice (27,28), we examined levels of neutral lipids in adrenal glands of *Gipr*$^{-/-}$ mice using Oil Red O histochemistry. The intensity of Oil Red O staining in the adrenal cortex was clearly reduced after 9 weeks of a HF diet in *Gipr*$^{-/-}$ mice (Fig. 5*B*). Conversely, activation of the GIPR with [D-Ala^2]GIP significantly increased incorporation of Oil Red O in Y1GIPR cells (Fig. 5*C*).

[D-Ala^2]GIP directly stimulates steroidogenesis and increases expression of steroidogenic genes in Y1 cells. As native Y1 adrenocortical cells do not express an endogenous GIPR (Supplementary Fig. 1), we used Y1 mouse adrenocortical cells stably transfected with the rat GIPR cDNA. Y1GIPR cells exhibited a robust dose-dependent increase in cAMP levels in response to GIP (Fig. 6*A*). In a similar manner, ERK1/2 phosphorylation was rapidly increased after exposure to [D-Ala^2] GIP ($P < 0.05$) (Fig. 6*B*). Because Y1 cells do not produce corticosterone (29), we assessed progesterone secretion in response to [D-Ala^2]GIP. A fivefold increase in progesterone secretion was observed after treatment with [D-Ala^2]GIP ($P < 0.001$) (Fig. 6*C*). Steroidogenesis is under tight control by StAR, a protein that transports cholesterol from the outer to inner mitochondrial membrane to Cyp11A1, which cleaves the cholesterol side chain to initiate the rate-limiting step of corticosterone synthesis. Therefore, we assessed whether [D-Ala^2]GIP regulates this pathway in Y1GIPR cells. [D-Ala^2]GIP significantly increased *StAR*, *cyp11a1*, and *sr-b1* mRNA transcript levels and increased *sf-1* transcript levels (Fig. 6*D*). This stimulatory effect of [D-Ala^2]GIP on gene expression was not significant for *pp1a* (cyclophillin), *mc2r*, *ldlr*, or *acat*.

Corticosterone supplementation does not exacerbate body weight gain in *Gipr*$^{-/-}$ mice. To determine if lower corticosterone levels in *Gipr*$^{-/-}$ mice contribute to their

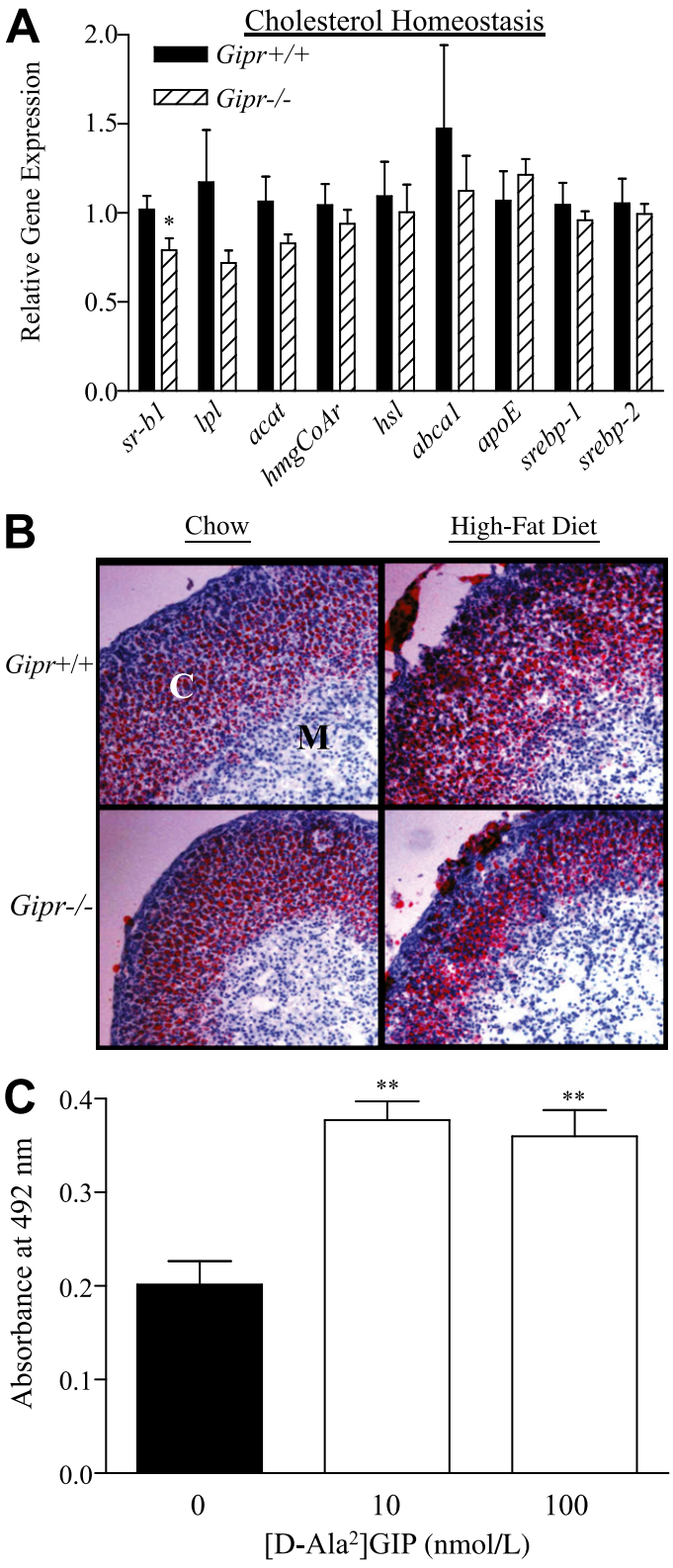

FIG. 5. GIP regulates lipid accumulation in adrenocortical cells. *A*: Expression of genes involved in adrenal cholesterol homeostasis was measured in adrenal glands from *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice by real-time qRT-PCR after 2 weeks HF feeding (n = 8–9 per group). *B*: The intensity of Oil Red O staining for neutral lipids is reduced in adrenal glands from *Gipr*$^{-/-}$ mice compared with *Gipr*$^{+/+}$ littermates after 9 weeks of HF feeding (n = 3 per group; original magnification ×10). C, cortex; M, medulla. *C*: Elution of Oil Red O from Y1GIPR adrenocortical cells is increased after 7 days treatment with [D-Ala^2]GIP (n = 3 per group). *P < 0.05 vs. *Gipr*$^{+/+}$ mice, **P < 0.01 vs. saline control. (A high-quality digital representation of this figure is available in the online issue.)

metabolic phenotype, *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice were supplemented with 5 μg/mL corticosterone in their drinking water and fed a HF diet for 22 weeks. Water and corticosterone intake were not affected by genotype (data not shown). Adrenal weights after 22 weeks were not different between vehicle-treated *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice. However, adrenal weights (Fig. 7*A*) were significantly reduced in both genotypes as a result of corticosterone supplementation ($P < 0.05$), consistent with feedback inhibition of the HPA axis from exogenous corticosterone administration. Body weights increased over time with HF feeding (Fig. 7*B*), and *Gipr*$^{-/-}$ mice gained less weight than wild-type littermates. However, weight gain was not affected by corticosterone supplementation in *Gipr*$^{+/+}$ versus *Gipr*$^{-/-}$ mice. Nocturnal food intake was slightly reduced in *Gipr*$^{-/-}$ mice ($P < 0.02$), as was ad libitum food intake ($P = 0.02$) (Supplementary Fig. 2), although this was no longer significant when normalized to body weight. Food intake was not altered by corticosterone supplementation. Locomotor activity was increased in *Gipr*$^{-/-}$ mice during the dark phase compared with *Gipr*$^{+/+}$ mice; however, neither V_{O_2} nor locomotor activity was affected by corticosterone supplementation (Fig. 7*C* and *D*). *Gipr*$^{-/-}$ mice had reduced fat mass; however, corticosterone supplementation had no effect on fat (Fig. 7*E*) or lean mass (data not shown) in *Gipr*$^{-/-}$ mice.

Corticosterone supplementation does not impair glucose homeostasis in *Gipr*$^{-/-}$ mice. Overnight fasting glucose levels (~16-h fast) were not different between genotypes or affected by corticosterone supplementation (Fig. 8*A* and *B*). Corticosterone supplementation had no effect on oral or intraperitoneal glucose tolerance in *Gipr*$^{+/+}$ or *Gipr*$^{-/-}$ mice (Fig. 8*A* and *B*). Despite comparable glucose levels, insulin levels (Fig. 8*A* and *B*) and insulin-to-glucose ratios ($P < 0.05$) (data not shown) to oral but not intraperitoneal glucose were reduced in *Gipr*$^{-/-}$ mice. Insulin sensitivity assessed by ITT was neither different between genotypes nor affected by corticosterone supplementation (Fig. 8*C*).

DISCUSSION

We have shown that activation of the GIPR increases plasma glucocorticoid levels in mice and that GIP directly activates steroidogenic gene expression in mouse adrenocortical cells. It seems likely that GIP also promotes steroidogenesis via increasing uptake of cholesterol since *Gipr*$^{-/-}$ mouse adrenal glands expressed lower levels of *sr-b1* mRNA transcripts and had reduced neutral lipid staining, whereas Y1GIPR cells stimulated with [D-Ala^2]GIP expressed higher transcript levels of *sr-b1* and stored more neutral lipid. Since glucocorticoids are diabetogenic and obesigenic in rodents (16,18), we hypothesized that reduced glucocorticoid levels in *Gipr*$^{-/-}$ mice might contribute to their resistance to diet-induced obesity and preservation of insulin sensitivity and glucose tolerance observed after HF feeding. However, supplementation of drinking water with low-dose corticosterone did not alter energy balance, insulin sensitivity, or glucose tolerance in HF-fed *Gipr*$^{-/-}$ mice.

Pharmacological levels of GIP stimulate ACTH secretion from AtT20 mouse corticotrope cells (30), and the *Gipr* is expressed in the hypothalamus and pituitary (11). Although HF-fed *Gipr*$^{-/-}$ mice exhibited a twofold reduction in basal corticosterone levels, plasma ACTH, pituitary *pomc*, and hypothalamic *crh* mRNA levels were not different.

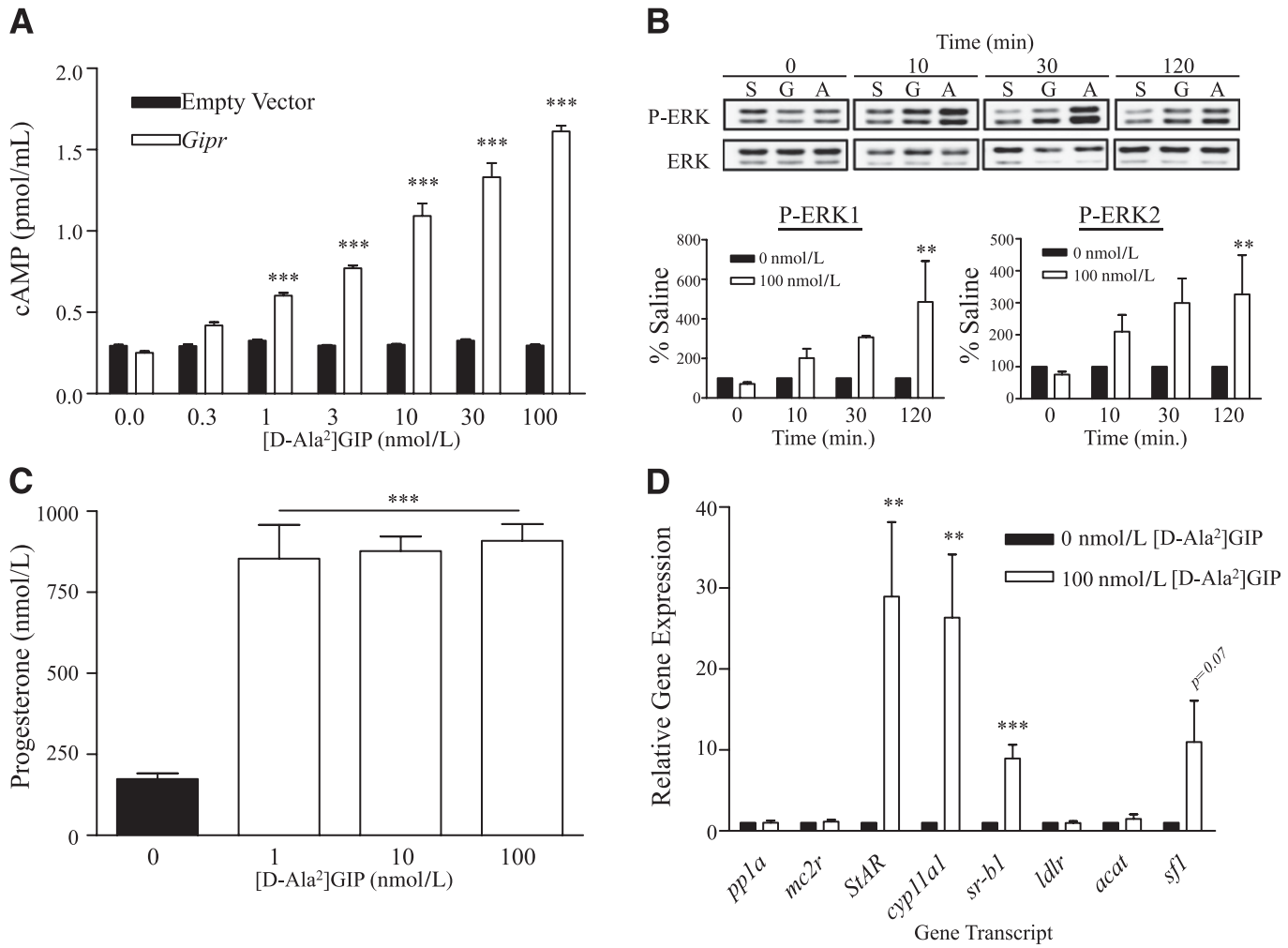

FIG. 6. [D-Ala2]GIP stimulates steroidogenesis in Y1 cells. *A*: GIP stimulates an increase in cAMP production by Y1 mouse adrenocortical cells stably transfected with the GIPR in a dose-dependent manner. *B*: Incubation of 72-h serum-starved Y1GIPR cells with 100 nmol/L [D-Ala^2]GIP increases ERK1/2 phosphorylation (P-ERK1/2; n = 6 per group). S, saline; G, [D-Ala^2]GIP; A, ACTH. *C*: Treatment (24 h) of Y1 cells with [D-Ala^2]GIP stimulates progesterone production. *D*: Incubation (6 h) of Y1 adrenocortical cells with [D-Ala^2]GIP increases *StAR*, *cyp11a1*, and *sr-b1* mRNA transcripts measured by real-time qRT-PCR (n = 5–7 per group). **P < 0.01 vs. saline, ***P < 0.001 vs. empty vector/saline.

Nevertheless, the increased adrenal expression of the ACTH receptor and enhanced adrenal sensitivity to ACTH in adrenal glands from *Gipr*$^{-/-}$ mice suggest the evolution of adaptive compensatory mechanisms that mask the impact of loss of adrenal GIP action. Enhanced ACTH sensitivity in *Gipr*$^{-/-}$ mice likely diminishes the extent of corticosterone deficiency and metabolic effects that might otherwise arise pursuant to elimination of GIPR action in the adrenal gland.

Further evidence for a direct effect of GIP on the adrenal gland was obtained in experiments using Y1GIPR cells. [D-Ala^2]GIP directly stimulated steroidogenesis, increased cAMP, activated the ERK pathway, and increased *StAR*, *cyp11a1*, *sf-1*, and *sr-b1* gene expression. Conversely, mRNA levels of *sf-1*, a transcription factor critical for regulation of adrenal steroidogenesis (31), and levels of *sr-b1*, a downstream target of SF-1 (32), were reduced in adrenal glands from *Gipr*$^{-/-}$ mice. GIP likely mediates some of its effects on the adrenal gland via modifying cholesterol uptake and/or storage. *Gipr*$^{-/-}$ mice had less neutral lipid staining in the adrenal cortex after 9 weeks of HF feeding, whereas [D-Ala^2]GIP increased neutral lipid accumulation in Y1GIPR cells. Since *sr-b1* mediates selective uptake of cholesterol from HDL, the most important pathway for adrenal cholesterol uptake in mice, and depletion of *sr-b1* leads to deficits in corticosterone (28,33), it is likely that GIP mediates adrenal steroidogenesis in part via this cholesterol uptake pathway.

Although the corticosterone responses to restraint stress, insulin-induced hypoglycemia, and high-dose ACTH were normal in *Gipr*$^{-/-}$ mice, the more chronic stressor of 24-h food deprivation elicited a threefold greater corticosterone response in *Gipr*$^{-/-}$ mice. In contrast to potent stressors such as restraint and insulin, food deprivation does not significantly increase ACTH levels, but it does increase basal corticosterone levels by reducing hepatic glucocorticoid clearance (34,35). Hence, at the low ACTH levels associated with food deprivation, the greater adrenal sensitivity to ACTH in *Gipr*$^{-/-}$ mice may be unmasked, leading to differentially greater increases in corticosterone responses in *Gipr*$^{-/-}$ mice. In contrast, no differences in corticosterone levels are apparent under situations where ACTH levels would already be expected to be high, as exemplified by restraint and insulin. Thus, it is likely that food deprivation is associated with reduced hepatic clearance of corticosterone and enhanced sensitivity to

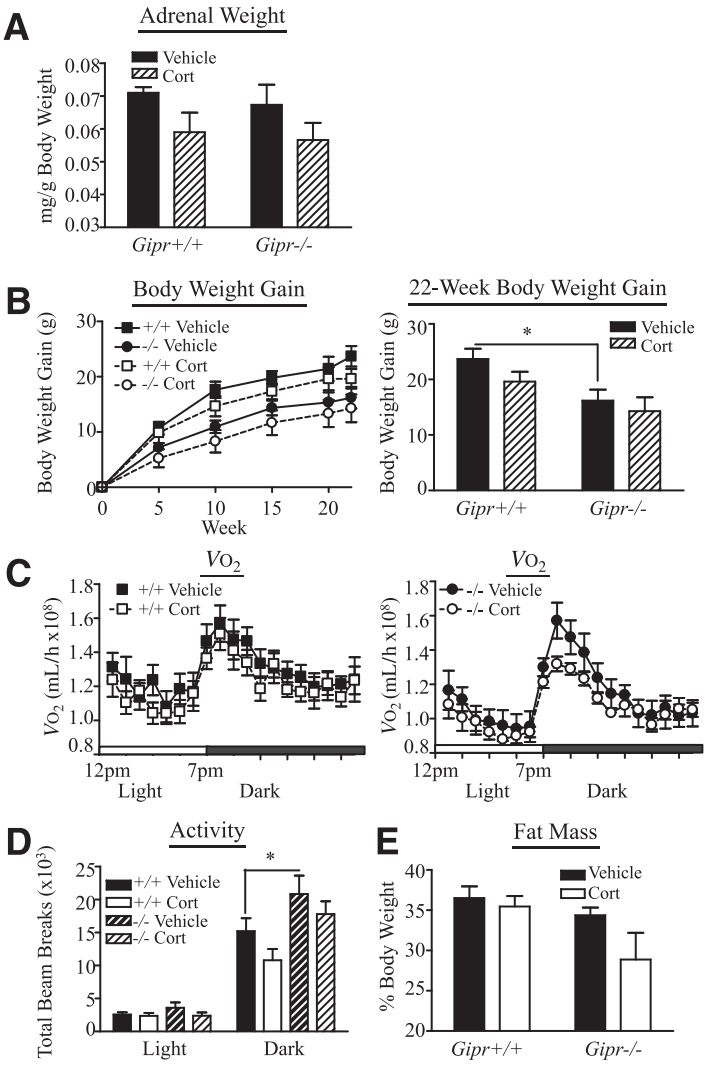

FIG. 7. Corticosterone supplementation does not alter energy balance in *Gipr*$^{-/-}$ mice. HF-fed *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice were supplemented with 5 μg/mL corticosterone in the drinking water for 22 weeks. *A*: Adrenal gland weights were reduced in mice supplemented with corticosterone in their drinking water (main effect corticosterone, $P < 0.05$). *B*: *Gipr*$^{-/-}$ mice gained weight more slowly than wild-type littermates, but this weight gain was not affected by corticosterone supplementation. Vo_2 (*C*) was not altered, whereas activity (*D*) was increased in *Gipr*$^{-/-}$ mice compared with wild-type littermates during the beginning of the dark phase (main effect genotype, $P < 0.05$). *E*: Assessment of fat mass by magnetic resonance imaging after 16 weeks of corticosterone supplementation and HF feeding demonstrated that *Gipr*$^{-/-}$ mice had reduced fat mass (main effect genotype, $P = 0.03$), which was not altered by corticosterone supplementation (n = 7–8 per group). *$P < 0.05$ vs. *Gipr*$^{+/+}$. CORT, corticosterone supplementation.

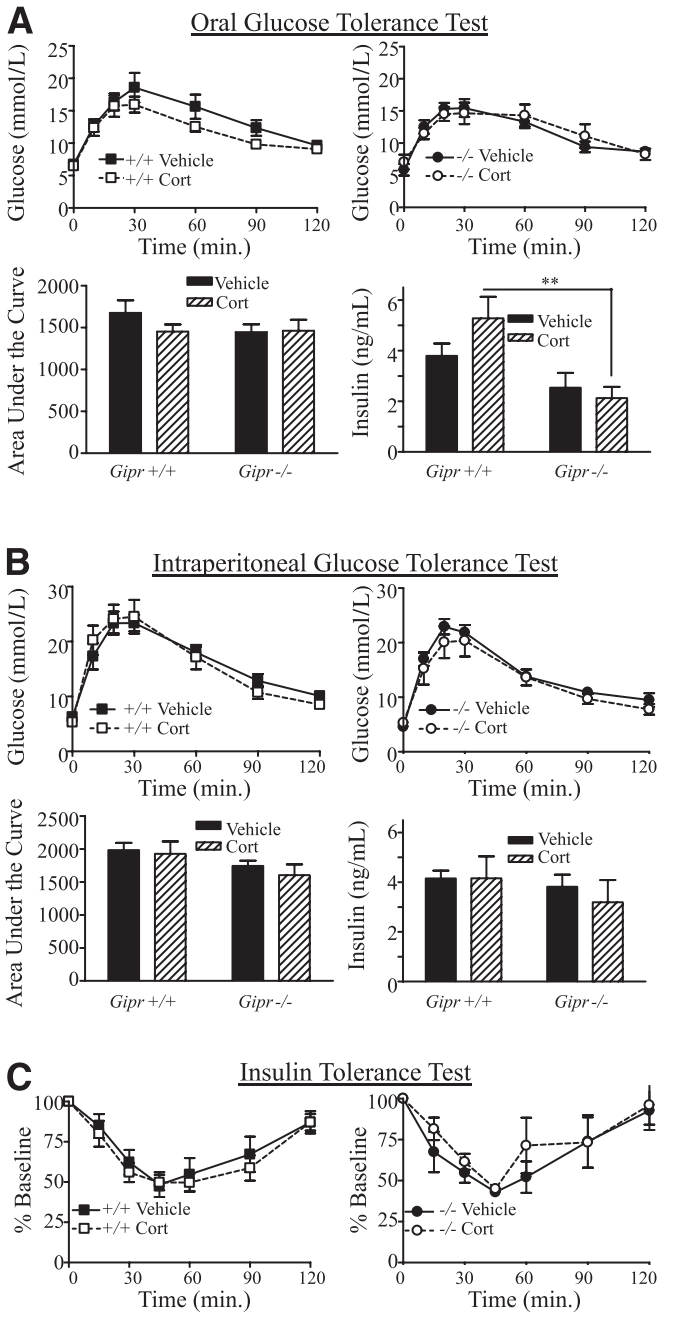

FIG. 8. Corticosterone supplementation does not worsen glucose homeostasis in *Gipr*$^{-/-}$ mice. Glucose excursion and 20–30 min plasma insulin responses after oral gavage (n = 7–8) (*A*) and intraperitoneal injection (n = 4–7) (*B*) of 1.5 mg/g glucose. Corticosterone supplementation reduced glucose excursion after oral glucose in wild-type mice (main effect corticosterone, $P = 0.03$) but not in *Gipr*$^{-/-}$ mice. Plasma insulin was reduced at 20–30 min in *Gipr*$^{-/-}$ mice after oral but not intraperitoneal glucose (n = 7–8). Insulin sensitivity (n = 6–7) (*C*) assessed by ITT was not affected by corticosterone supplementation in *Gipr*$^{+/+}$ vs. *Gipr*$^{-/-}$ mice. CORT, corticosterone supplementation.

lower levels of ACTH, leading to hypercorticosteronemia in *Gipr*$^{-/-}$ mice.

Glucocorticoids regulate metabolism through stimulation of hepatic glucose production (36), impairment of insulin sensitivity and glucose tolerance (37), inhibition of insulin secretion (38), and facilitation of lipolysis (23). Chronic elevations of glucocorticoids promote fat deposition (23,39), insulin resistance (39), hepatosteatosis, hyperphagia, and decreased locomotion (39). Many commonly used rodent models of obesity or insulin resistance, including *ob/ob* and *db/db* mice, Zucker and ZDF rats, HF-fed rodents, and streptozotocin-induced diabetic rodents, exhibit elevations in glucocorticoids (14–17). Adrenalectomy or administration of a glucocorticoid antagonist can ameliorate or in some cases reverse the abnormal metabolic phenotype (18,19), illustrating the importance of glucocorticoids in the development of rodent obesity and diabetes. Administration of a peptide GIPR antagonist in HF-fed mice lowers corticosterone levels in association with reduced body weight and fat deposition, increased locomotion, and improved glucose homeostasis (7,8,40). However, our current data in HF-fed *Gipr*$^{-/-}$ mice suggest that modest reductions in plasma corticosterone do not appear to substantially

modify the dominant metabolic phenotypes arising after HF feeding in *Gipr*$^{-/-}$ mice.

Corticosterone supplementation did not significantly modify food intake, energy expenditure, weight gain, or fat mass in *Gipr*$^{+/+}$ and *Gipr*$^{-/-}$ mice. However, corticosterone supplementation slightly improved oral but not intraperitoneal glucose tolerance in wild-type mice. This modest improvement was likely related to the small reductions in body weight and fat mass induced by corticosterone supplementation that would in turn improve glucose disposal. This reduction in fat mass may be related to the lipolytic effects of corticosterone at low concentrations (23). It is likely that the upregulation of ACTH sensitivity in *Gipr*$^{-/-}$ mice resulted in only a subtle reduction in plasma corticosterone that was not sufficient to lower weight gain or modify energy balance and glucose homeostasis. Consistent with our current data, Irwin et al. (7) demonstrated that administration of the GIP antagonist Pro(3)GIP to *ob/ob* mice for 60 days significantly improved glucose control and insulin sensitivity, without concomitant changes in circulating corticosterone.

In summary, we show that GIP stimulates plasma glucocorticoid levels in mice and demonstrate that GIP directly activates steroidogenesis through stimulation of steroidogenic genes as well as *sr-b1* expression and consequent adrenocortical lipid deposition. *Gipr*$^{-/-}$ mice compensate for disruption of GIP action in the adrenal gland via upregulation of *mcr2* expression and enhanced ACTH sensitivity. Corticosterone supplementation did not reverse the beneficial metabolic phenotype of HF-fed *Gipr*$^{-/-}$ mice, including resistance to diet-induced obesity and maintenance of oral glucose tolerance with lower levels of plasma insulin, implicating organs such as the brain and adipose tissue as potential mediators of the favorable *Gipr*$^{-/-}$ phenotype. Our findings may have relevance for envisioned therapeutic strategies using GIPR antagonists for the treatment of obesity and diabetes in human subjects. Because the GIP–adrenal axis does not appear to be functional in normal human subjects, the observation that manipulation of corticosterone levels does not substantially abrogate the beneficial metabolic phenotype of *Gipr*$^{-/-}$ mice has positive implications for targeting the GIPR in human subjects.

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H.E.B. was primarily responsible for the experimental design, experiments, interpretation, and writing of the manuscript. J.E.C., J.R.U., L.L.B., and A.M. contributed significantly to the experiments as well as data interpretation. D.J.D. contributed to the experimental design, interpretation of data, and writing of the manuscript and is the guarantor of the manuscript and takes responsibility for all aspects of the work.

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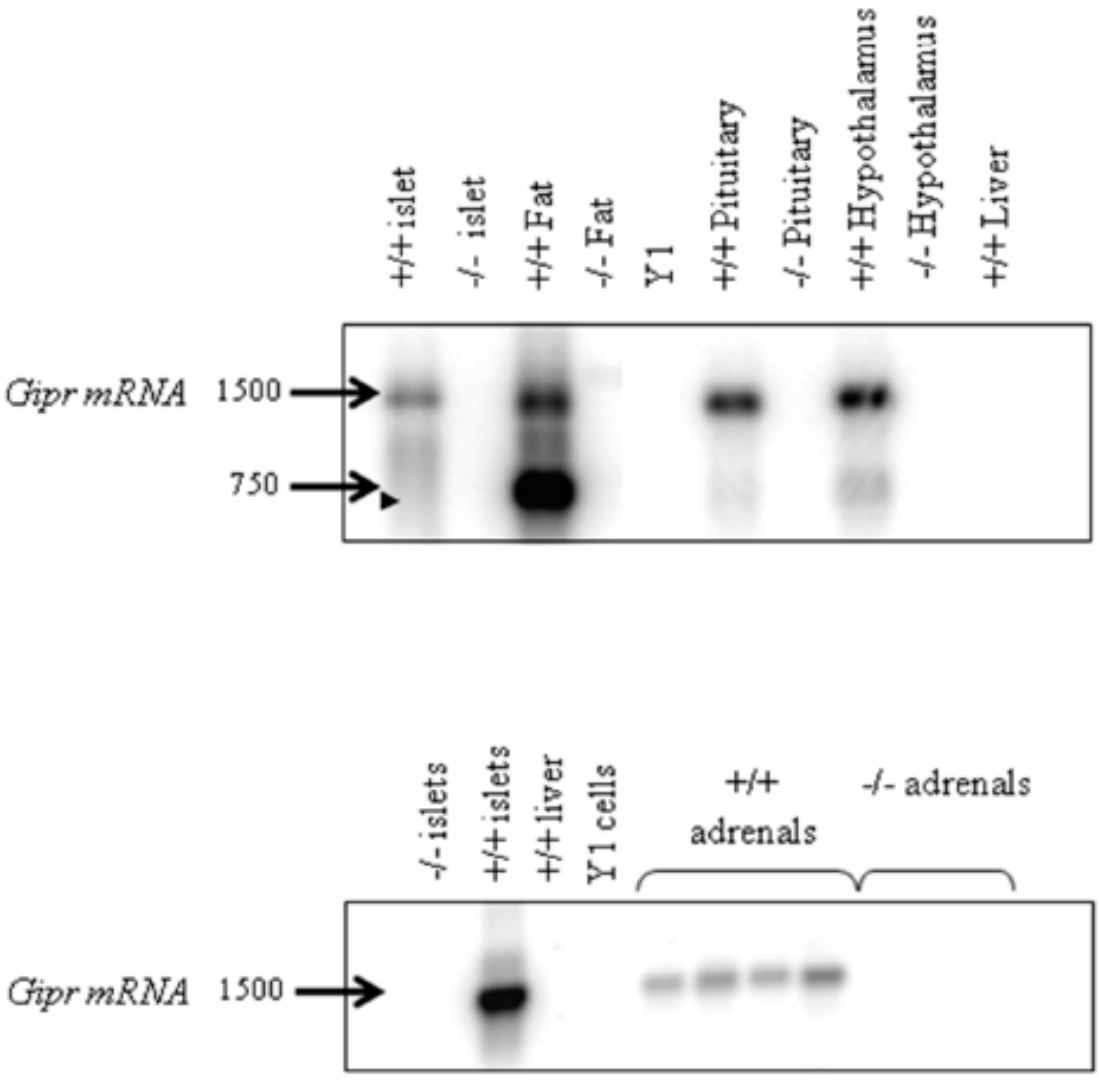
SUPPLEMENTARY DATA

Supplementary Figure 1. Representative blots demonstrating expression of *Gipr* mRNA in the pituitary, hypothalamus and adrenal gland in *Gipr*+/+ but not *Gipr*-/- mice.



SUPPLEMENTARY DATA

Supplementary Figure 2. Nocturnal food intake was slightly reduced in *Gipr*-/- mice (P<0.02) as was ad libitum food intake (P=0.02), although this was no longer significant when normalized to body weight. Food intake was not altered by corticosterone supplementation (Cort).