Insulin Action in the Double Incretin Receptor Knockout Mouse

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OBJECTIVE—The incretins glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide have been postulated to play a role in regulating insulin action, although the mechanisms behind this relationship remain obscure. We used the hyperinsulinemic-euglycemic clamp to determine sites where insulin action may be modulated in double incretin receptor knockout (DIRKO) mice, which lack endogenous incretin action.

RESEARCH DESIGN AND METHODS—DIRKO and wild-type mice were fed regular chow or high-fat diet for 4 months. Clamps were performed on 5-h–fasted, conscious, unrestrained mice using an arterial catheter for sampling.

RESULTS—Compared with wild-type mice, chow and high fat–fed DIRKO mice exhibited decreased fat and muscle mass associated with increased energy expenditure and ambulatory activity. Clamp rates of glucose infusion (GIR), endogenous glucose production (endoR_a), and disappearance (R_d) were not different in chow-fed wild-type and DIRKO mice, although insulin levels were lower in DIRKO mice. Liver Akt expression was decreased but Akt activation was increased in chow-fed DIRKO compared with wild-type mice. High-fat feeding resulted in fasting hyperinsulinemia and hyperglycemia in wild-type but not in DIRKO mice. GIR, suppression of endoR_a, and stimulation of R_d were inhibited in high fat–fed wild-type mice but not in DIRKO mice. High-fat feeding resulted in impaired tissue glucose uptake (R_g) in skeletal muscle of wild-type mice but not of DIRKO mice. Liver and muscle Akt activation was enhanced in high fat–fed DIRKO compared with wild-type mice.

CONCLUSIONS—In summary, DIRKO mice exhibit enhanced insulin action compared with wild-type mice when fed a regular chow diet and are protected from high-fat diet–induced obesity and insulin resistance. *Diabetes* **57:288–297, 2008**

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After ingestion of a meal, multiple hormones are secreted from specialized enteroendocrine cells in the gut epithelium that coordinate events necessary for proper absorption and storage of nutrients. One such event is the control of glucose excursions and maintenance of glucose homeostasis after nutrient ingestion. Two gut-derived incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), play a significant role in this process by enhancing insulin secretion from pancreatic β-cells in response to oral nutrient intake (1,2). This incretin effect is mediated via activation of distinct G-protein–coupled receptors for GIP (GIPR) and GLP-1 (GLP-1R) in β-cells, which initiates signaling cascades necessary for glucose-dependent insulin secretion (1).

GIP and GLP-1 also regulate glucose homeostasis via actions independent of insulin secretion. Activation of the incretin receptors stimulate pathways that enhance β-cell proliferation and inhibit β-cell apoptosis (3–7). GLP-1 inhibits glucagon secretion from pancreatic α-cells and delays gastric emptying, thus preventing large excursions in blood glucose levels (8–10). GLP-1 may also exert control on hepatic glucose production and peripheral glucose clearance independent of its effects on insulin secretion (11,12). GIP has been shown to stimulate insulin-mediated glucose uptake and lipoprotein lipase activity in cultured adipocytes (13).

Mice lacking either GIPR or GLP-1R exhibit only mild glucose intolerance due to impaired glucose-stimulated insulin secretion (14–19). The absence of a more severe phenotype is likely due to compensatory increases in the circulating levels of and/or sensitivity to the remaining incretin hormone. For example, disruption of GLP-1R results in increased circulating levels of and enhanced sensitivity to GIP (20). Similarly, although GLP-1 levels do not increase in mice lacking GIPR, these mice exhibit enhanced sensitivity to GLP-1 action (21).

To understand the consequences of complete loss of incretin receptor signaling, double incretin receptor knockout (DIRKO) mice have been generated. Despite a complete absence of endogenous GIP and GLP-1 action, DIRKO mice exhibit only moderate glucose intolerance compared with single incretin receptor knockout mice (22,23). Despite the metabolic stress imposed by chronic high-fat feeding, DIRKO mice chronically fed a high-fat diet exhibit only a modest deterioration in glucose tolerance, together with resistance to diet-induced obesity and increased energy expenditure and locomotor activity (24). The aims of the present studies are 1) to assess the effects of a complete disruption of incretin signaling on insulin action in regular chow-fed mice and 2) to determine

whether the lack of incretin signaling impairs the normal compensatory mechanisms arising in high fat–fed mice. These aims were addressed using the hyperinsulinemic-euglycemic clamp, a technique that allows for the assessment of insulin action in conscious, unrestrained mice.
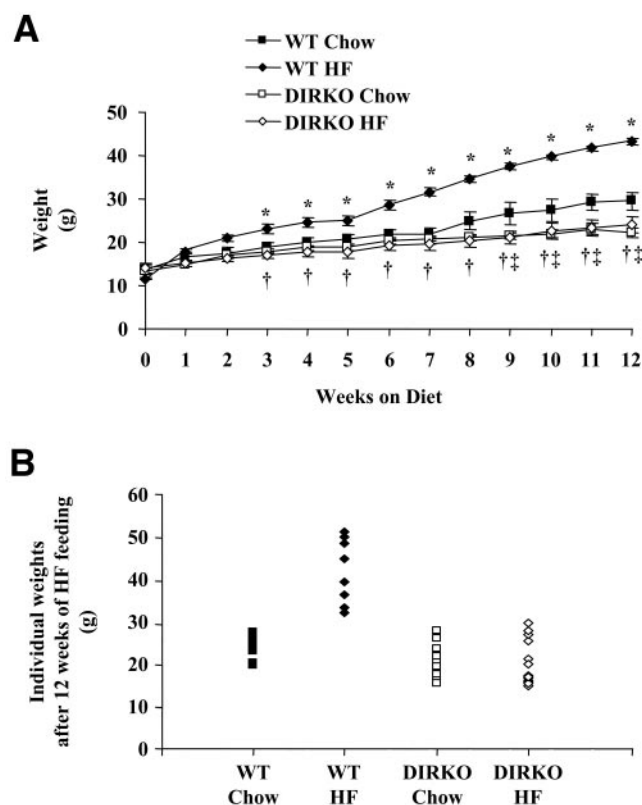
RESEARCH DESIGN AND METHODS

Mouse maintenance and genotyping. All procedures were approved by the Vanderbilt Animal Care and Use Committee. DIRKO mice on a CD1 background were backcrossed onto a C57BL/6 background (at least 10 generations) and bred to C57BL/6 mice (The Jackson Laboratories) to obtain double incretin receptor heterozygous founder mice. A heterozygous breeding pair was then used to generate wild-type and DIRKO breeding pairs, such that all subsequent wild-type and DIRKO mice used in these studies originated from the same heterozygous founder breeding pair. Littermates were weaned and separated by sex at 3 weeks of age. Genotyping was done by PCR of genomic DNA obtained from a tail biopsy. All mice were fed either regular chow (Purina 5001; Purina Mills) or high-fat diet (F-3282; Bio-Serv) ad libitum, were handled at least once per week, and were studied at ~4 months of age. Regular chow diet contains 12.1% calories from fat, whereas high-fat diet contains 59.4% calories from fat.

Assessment of body composition, feeding, and energy expenditure. Body composition was determined at ~4 months of age using an mq10 NMR analyzer (Bruker Optics). For food intake assessment, mice were placed in individual cages with measured amounts of food and bedding. The remaining food was weighed 48 h later, excluding fecal matter and bedding. Oxygen consumption (Vo_2) and carbon dioxide production (Vco_2) were measured using an Oxymax indirect calorimetry system (Columbus Instruments) as described previously (25). Energy expenditure was calculated as described previously (25,26). Respiratory exchange ratio (RER) was calculated as Vco_2/Vo_2. Ambulatory activity was estimated by the number of infrared beams broken in both X and Y directions.

Assessment of fat absorption. After 8 weeks of high-fat feeding, 20-week-old male wild-type and DIRKO mice ($n = 5$/group) were placed in individual cages with no bedding and provided with a preweighed amount of high-fat food. Every 24 h for 3 days, cages were changed, food was weighed, and total feces were collected. On completion of the collection phase, feces were completely dried to constant weight using a speed-vac at 60°C for 1 h. The mass of excreted feces was measured, and fat extraction was conducted on equal amounts of feces for both groups following the Nelson and O'Hopp method (27) with minor modifications. Briefly, feces were rigorously mixed in 1 volume of chloroform:methanol (1:2) and 2 volumes of 5 N HCl and incubated overnight in the dark at room temperature. After centrifugation for 30 min at 4,000 rpm, the organic phase was collected into preweighed tubes. The chloroform was evaporated, and tubes were reweighed to calculate fecal fat content.

Hyperinsulinemic-euglycemic clamps. Mice were catheterized at least 5 days before experimentation as described previously (28). Hyperinsulinemic-euglycemic clamps were performed on 5-h–fasted mice (28). A 5-μCi bolus of [3-^{3}H]glucose was given at $t = -90$ min before insulin infusion, followed by a 0.05 μCi/min infusion for 90 min. Blood samples were obtained via an arterial catheter (28). Basal glucose specific activity was determined from blood samples at $t = -15$ and -5 min. Fasting insulin levels were determined from blood samples taken at $t = -5$ min. The clamp was begun at $t = 0$ min with a continuous infusion of human insulin (4 mU · kg^{-1} · min^{-1}, Humulin R; Eli Lilly). The [3-^{3}H]glucose infusion was increased to 0.2 μCi/min for the remainder of the experiment. Euglycemia (~150–160 mg/dl) was maintained by measuring blood glucose every 10 min starting at $t = 0$ min and infusing 50% dextrose as necessary. Mice received saline-washed erythrocytes from donors throughout the clamp (5–6 μl/min) to prevent a fall of >5% hematocrit. A 12-μCi bolus of 2[^{14}C]deoxyglucose (2[^{14}C]DG) was given at $t = 78$ min. Blood samples (80–240 μl) were taken every 10 min from $t = 80$ to 120 min and processed to determine plasma [3-^{3}H]glucose and 2[^{14}C]DG. Clamp insulin

FIG. 1. Weight gain in chow-fed and high fat–fed C57BL/6 (wild-type) and DIRKO mice. Chow-fed wild type (■), high fat–fed wild type (♦), chow-fed DIRKO (□), high-fat-fed DIRKO (◊). _A_: Growth curve during the 12-week feeding period. _B_: Individual weights at the end of the 12-week feeding period. Data are mean ± SE for 8–15 mice/group. *_P_ < 0.05 vs. chow; †_P_ < 0.05 DIRKO high fat vs. wild-type high fat; ‡_P_ < 0.05 DIRKO chow vs. wild-type chow.

was determined at $t = 100$ and 120 min. At $t = 120$ min, mice were anesthetized with sodium pentobarbital. The soleus, gastrocnemius, superficial vastus lateralis (SVL), liver, diaphragm, heart, and brain were excised, immediately frozen, and stored at −80°C until analyzed.

Processing of plasma and muscle samples. Insulin levels were determined by ELISA (Linco). Nonesterified fatty acids (NEFAs) were measured spectrophotometrically by an enzymatic colorimetric assay (Wako NEFA HR2 kit; Wako Chemicals). After deproteinization with barium hydroxide [Ba(OH)$_2$, 0.3 N] and zinc sulfate [ZnSO$_4$, 0.3 N], plasma [3-^{3}H]glucose and 2[^{14}C]DG radioactivity was determined by liquid scintillation counting (Packard TRI-CARB 2900TR) with Ultima Gold (Packard) as scintillant. Muscle samples were weighed and homogenized in 0.5% perchloric acid. Homogenates were centrifuged and neutralized with KOH. One aliquot was counted directly to determine 2[^{14}C]DG and 2[^{14}C]DG-6-phosphate (2[^{14}C]DGP) radioactivity. A second aliquot was treated with Ba(OH)$_2$ and ZnSO$_4$ to remove 2[^{14}C]DGP and any tracer incorporated into glycogen and then counted to determine 2[^{14}C]DG radioactivity. 2[^{14}C]DGP is the difference between the two aliquots. In all experiments, the accumulation of 2[^{14}C]DGP was normalized to tissue weight.
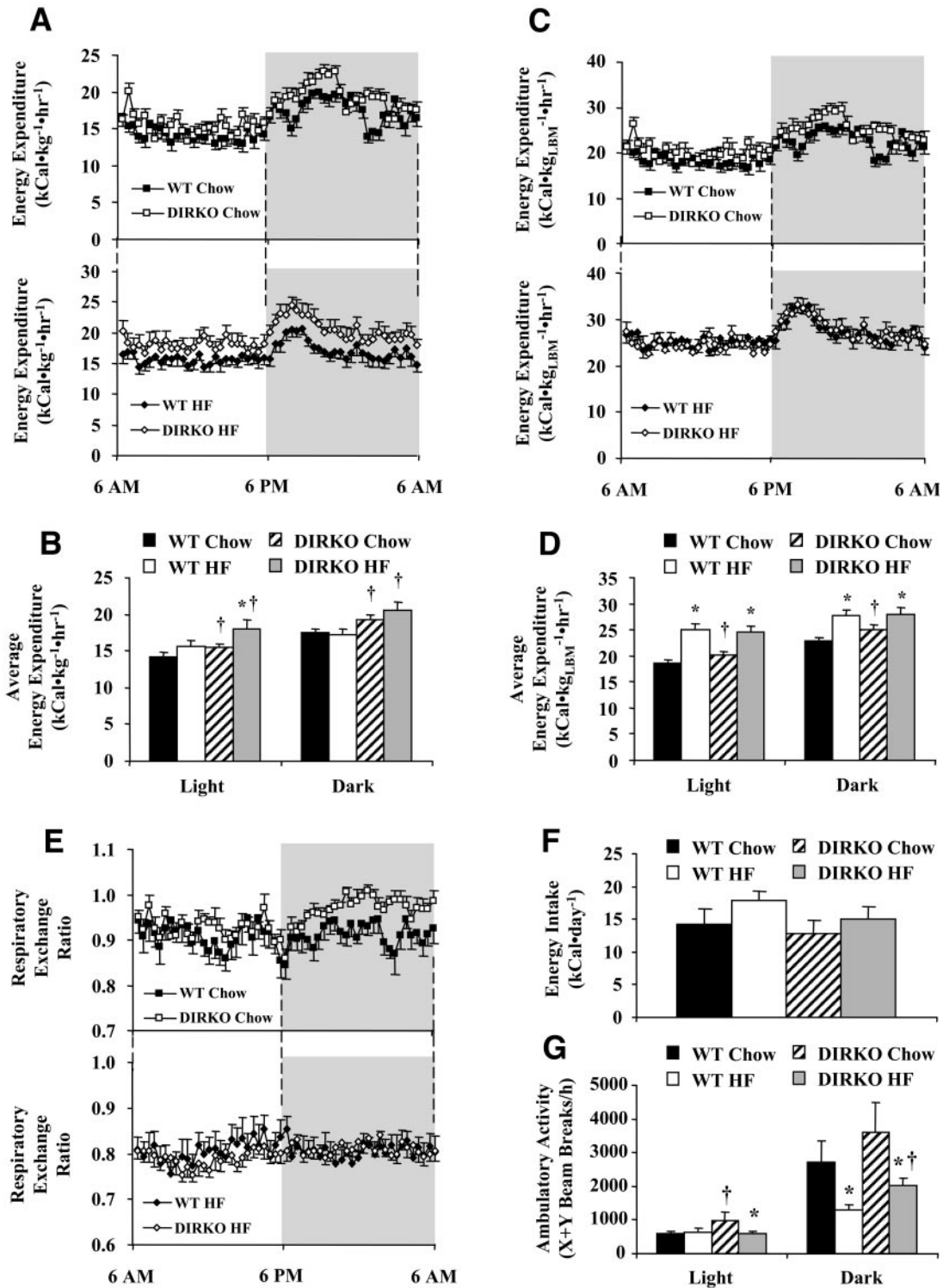
Isolation of whole-cell extracts and immunoblotting. Liver and muscle (gastrocnemius) tissue (20–40 mg) was homogenized in 10 μl/mg tissue extraction buffer (50 mmol/l Tris, 1 mmol/l EDTA, 1 mmol/l EGTA, 10% glycerol, and 1% Triton-X 100, pH 7.5) supplemented with protease (Pierce)

TABLE 1
Body composition in chow-fed and high fat–fed C57BL/6 (wild-type) and DIRKO mice

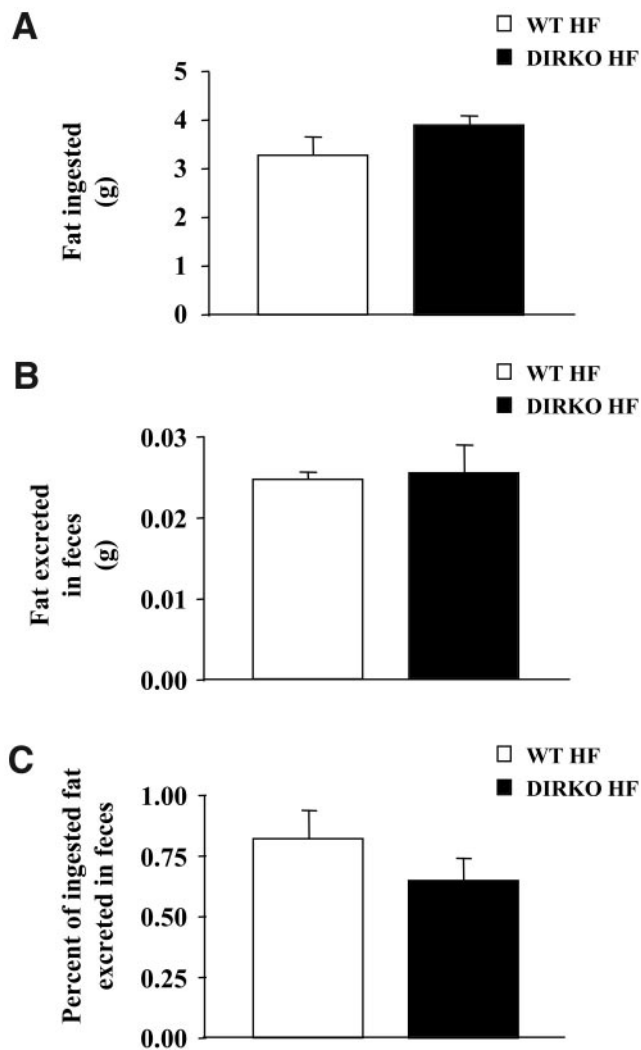|	Wild-type chow fed	Wild-type high-fat fed	DIRKO chow fed	DIRKO high-fat fed
n (males/females)	12 (6/6)	8 (5/3)	13 (6/7)	15 (9/6)
Weight (g)	23.6 ± 0.8	41.9 ± 2.7*	21.1 ± 1.0†	22.1 ± 1.3†
Fat (g)	2.0 ± 0.1	14.2 ± 2.1	2.1 ± 0.1	3.8 ± 0.7
Muscle (g)	18.2 ± 0.6	24.5 ± 1.0	16.2 ± 0.8†	15.9 ± 0.6

Data are means ± SE. *P < 0.05 vs. chow; †P < 0.05 vs. wild type.

FIG. 2. Energy expenditure and intake measurements in chow-fed and high fat–fed C57BL/6 (wild-type) and DIRKO mice. Black squares and black bars, chow-fed wild type; black diamonds and white bars, high fat–fed wild type; white squares and striped bars, chow-fed DIRKO; white diamonds and gray bars, high fat–fed DIRKO. A: Energy expenditure normalized to total body weight. B: Average energy expenditure normalized to total body weight. C: Energy expenditure normalized to total LBM. D: Average energy expenditure normalized to LBM. E: RER. F: Average energy intake. G: Average ambulatory activity estimated as X+Y beam breaks over a 24-h period. Data are means ± SE for 6–15 mice/group. *$P < 0.05$ vs. chow; †$P < 0.05$ vs. wild type.

and phosphatase (Sigma) inhibitor cocktails. Homogenates were centrifuged (20 min, 4,500g, 4°C), pellets were discarded, and supernatants were retained for protein determination. Protein content was determined using a BCA protein assay kit (Bio-Rad). Whole-cell (20-µg) extracts were separated on 10% Bis-Tris SDS-PAGE gels (Invitrogen), followed by electrophoretic transfer to polyvinylidine fluoride membranes. Primary antibodies were

incubated with the membranes overnight at 4°C. Secondary antibodies were incubated at room temperature for 1 h. Imaging and densitometry were performed using the Odyssey imaging system (Li-Cor). Rabbit anti-Akt (1:1,000) and anti-phospho(Ser473)-Akt (1:1,000) were from Cell Signaling. Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (1:2,000) was from Abcam.

FIG. 3. Ingestion and fecal excretion of fat in high fat–fed C57BL/6 (wild-type) and DIRKO mice. □, high fat–fed wild type; ▨, high fat–fed DIRKO. A: Total fat ingested over a 72-h period. B: Total fat excreted in feces over a 72-h period. C: Percent of total fat ingested excreted in feces over a 72-h period. Data are means ± SE for five mice/group.

Calculations. Glucose appearance (R_a) and disappearance (R_d) were determined using Steele nonsteady-state equations (29). Endogenous glucose production (endoR_a) was determined by subtracting the glucose infusion rate (GIR) from total R_a. Glucose metabolic index (R_g) was calculated as previously described (25,30,31). R_g for all tissues was normalized to brain R_g.

Statistical analysis. Data are presented as means ± SE. Differences between groups were determined by two-way ANOVA followed by Tukey's post hoc tests or by one-tailed t test as appropriate. The significance level was $P < 0.05$.
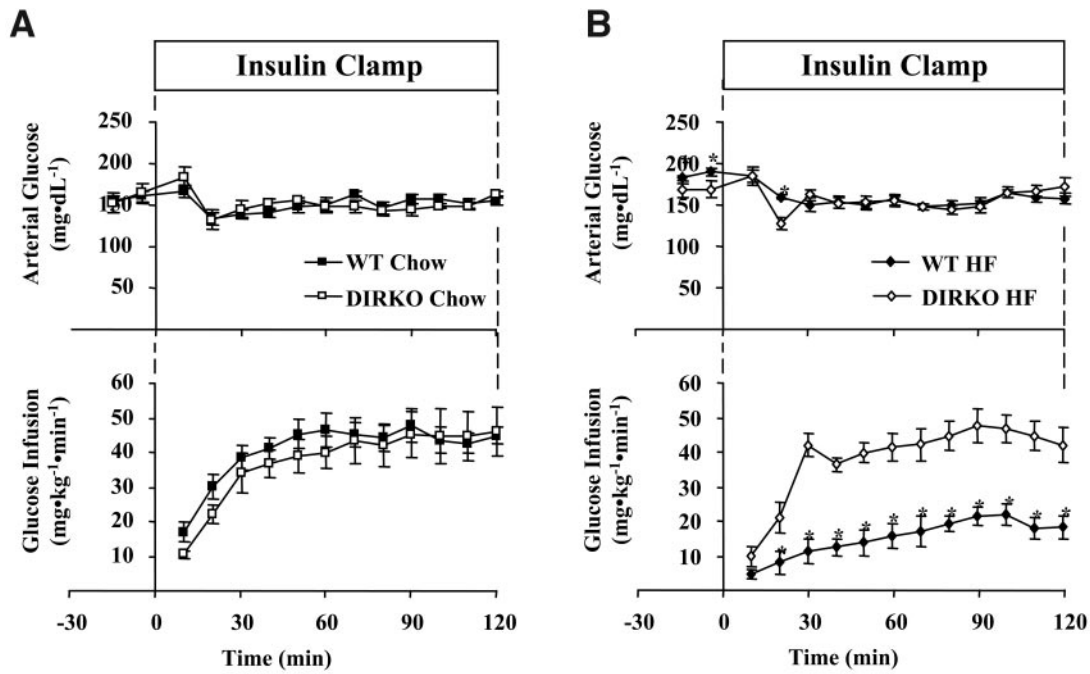
RESULTS

DIRKO mice exhibit decreased weight gain and fat mass due to increased energy expenditure and activity. DIRKO mice on a C57BL/6 background and wild-type mice were placed on a regular chow or a high-fat diet for 12 weeks beginning at 3 weeks of age. DIRKO mice had decreased weight gain associated with reduced muscle mass compared with wild-type mice on a chow diet (Fig. 1; Table 1). High-fat feeding resulted in increased weight gain in wild-type mice (Fig. 1) associated with increased fat and muscle mass (Table 1). In contrast, high-fat feeding did not affect weight gain in DIRKO mice (Fig. 1) and resulted in only a small increase in fat mass (Table 1).

Energy expenditure normalized to total body weight was increased in DIRKO mice compared with wild type on both chow and high-fat diets (Fig. 2A and B). DIRKO mice on high-fat diet also exhibited higher light-cycle energy expenditure than DIRKO mice on chow diet. When normalized to lean body mass, energy expenditure was increased in DIRKO mice compared with wild-type mice on chow but not on high-fat diet (Fig. 2D). RER was also not different between wild-type and DIRKO mice on either diet (Fig. 2E). Energy intake was not different between wild-type and DIRKO mice on either diet (Fig. 2F). Light-cycle ambulatory activity was increased in chow-fed, but not high fat–fed, DIRKO mice compared with wild-type mice (Fig. 2G). Conversely, dark-cycle ambulatory activity was higher in high fat–fed, but not chow-fed, DIRKO mice compared with wild-type mice (Fig. 2G). High-fat–fed DIRKO mice did not exhibit defective fat absorption, because fat intake and excretion were normal compared with high fat–fed wild-type mice (Fig. 3).

Chow- and high fat–fed DIRKO mice exhibit enhanced whole-body insulin action. Insulin action was assessed in chow-fed, conscious, unrestrained wild-type and DIRKO mice using the hyperinsulinemic ($4 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)-euglycemic clamp. Fasting (5-h fast) glucose and insulin levels were not different between wild-type and DIRKO mice (Fig. 4A, *top panel*; Table 2). Fasting NEFAs were lower in DIRKO mice (Table 2). Arterial glucose was clamped at similar levels in both groups (Fig. 4A, *top panel*). The GIR necessary to maintain euglycemia was not different between groups (Fig. 4A, *bottom panel*). However, clamp insulin levels were lower in DIRKO mice (Table 2). Thus, normalizing GIR to clamp insulin levels, an index of insulin sensitivity, shows enhanced insulin action in DIRKO mice (Table 2). Insulin-mediated suppression of endoR_a and stimulation of R_d were not different between wild-type and DIRKO mice (Fig. 5), although insulin levels were lower in the latter. Clamp NEFAs were significantly lower in DIRKO mice (Table 2). The ability of insulin to suppress NEFAs was not different between wild-type and DIRKO mice, as shown by the change in NEFAs normalized to the change in insulin from fasting to clamp levels (Table 2).

Insulin action was also assessed in high fat–fed wild-type and DIRKO mice. Fasting glucose and insulin levels were markedly higher in high fat–fed wild-type than DIRKO mice (Fig. 4B, *top panel*; Table 2). Compared with chow-fed mice, fasting insulin and glucose levels were higher in high fat–fed wild-type mice, indicative of fasting insulin resistance. By contrast, high-fat feeding had no effect on fasting glucose in DIRKO mice but resulted in markedly lower fasting insulin levels (Table 2). As in chow-fed mice, fasting NEFAs were lower in high fat–fed DIRKO mice, even though fasting insulin levels were markedly lower in this group (Table 2). Arterial glucose was clamped at similar levels in both high fat–fed wild-type and DIRKO mice (Fig. 4B, *top panel*). The GIR necessary to maintain euglycemia was significantly lower in wild-type mice (Fig. 4B, *bottom panel*), even as clamp insulin levels were higher in this group (Table 2). Thus, normalizing GIR to clamp insulin levels shows high fat–fed DIRKO mice to have a more than threefold higher index of insulin sensitivity than wild-type mice (Table 2). Furthermore, DIRKO mice are protected from high-fat diet–induced insulin resistance, because high-fat feeding resulted in a more than threefold decrease in insulin sensitivity in wild-type mice, whereas insulin sensitivity was insignificantly decreased in DIRKO mice (Table 2).

FIG. 4. Hyperinsulinemic-euglycemic clamps on 5-h–fasted, conscious, unrestrained chow-fed and high fat–fed C57BL/6 (wild-type) and DIRKO mice. *A*: Arterial glucose levels (*top panel*) and GIRs (*bottom panel*) during hyperinsulinemic-euglycemic clamps in chow-fed wild-type (■) and DIRKO (□) mice. *B*: Arterial glucose levels (*top panel*) and GIRs (*bottom panel*) during hyperinsulinemic-euglycemic clamps in high fat–fed wild-type (♦) and DIRKO (◇) mice. Data are means ± SE for 8–10 mice/group. *$P < 0.05$ vs. DIRKO.

Clamp NEFAs were also significantly lower in DIRKO mice (Table 2). As in chow-fed mice, suppression of NEFAs by insulin was not different between high fat–fed wild-type and DIRKO mice (Table 2).

Insulin-mediated suppression of endoR_a was not significantly different between high fat–fed wild-type and DIRKO mice, although insulin levels were drastically lower in the latter group (Fig. 5*A* and *B*). Compared with chow feeding, high-fat feeding resulted in impaired suppression of endoR_a in wild-type mice but not in DIRKO mice (Fig. 5*B*). Despite lower clamp insulin levels in DIRKO mice, stimulation of R_d was significantly higher in this group (Fig. 5*C* and *D*). Furthermore, whereas the stimulation of R_d was inhibited in high fat–fed wild-type mice, this inhibition did not occur in DIRKO mice.

Cardiac glucose uptake is enhanced in chow-fed DIRKO mice. A bolus of 2[^{14}C]DG was administered during the clamp to determine the glucose metabolism index (R_g), a measure of tissue glucose uptake. Absolute R_g in skeletal muscle was lower in chow-fed DIRKO mice compared with wild-type mice, as shown in gastrocnemius and SVL muscles (Fig. 6*A*). However, when normalized to clamp insulin levels, R_g in these muscles was not different between groups (Fig. 6*C*). Absolute R_g was not affected in oxidative muscles, such as soleus, diaphragm, and heart (Fig. 6*A* and *B*). When normalized to clamp insulin levels, R_g in the heart was increased in DIRKO compared with wild-type mice (Fig. 6*D*).

DIRKO mice are protected from high-fat diet–induced impairments in insulin-stimulated muscle glu-

TABLE 2
Fasting and clamp parameters in chow-fed and high fat–fed C57BL/6 (wild-type) and DIRKO mice

	Wild-type chow fed	Wild-type high-fat fed	DIRKO chow fed	DIRKO high-fat fed
n (males/females)	10 (6/4)	8 (4/4)	10 (5/5)	8 (4/4)
Glucose (mg/dl)				
Fasting	159 ± 5	185 ± 6*	159 ± 3	167 ± 6†
Clamp	154 ± 2	155 ± 3	150 ± 1	157 ± 5
Insulin (μU/ml)				
Fasting	42 ± 10	93 ± 22*	36 ± 7	16 ± 4*†
Clamp	85 ± 11	148 ± 25*	63 ± 4†	81 ± 8*†
NEFAs (mmol/l)				
Fasting	1.3 ± 0.1	1.4 ± 0.2	1.1 ± 0.1†	1.0 ± 0.1†
Clamp	0.6 ± 0.1	0.7 ± 0.1	0.4 ± 0.03†	0.5 ± 0.1†
GIR (mg · kg^{-1} · min^{-1})	45 ± 3	20 ± 7	45 ± 2	45 ± 4
Insulin sensitivity for glucose [mg · ml^{-1} · (kg · min^{-1} · mU^{-1})$^{-1}$]	546 ± 115	169 ± 79*	745 ± 79†	587 ± 92†
Insulin sensitivity for NEFA (Δmmol/l:ΔμU/ml)	1.7 ± 0.3	0.8 ± 0.3*	2.5 ± 0.4	0.8 ± 0.2*

Data are for 5-h–fasted, conscious, unrestrained mice at least 5 days after surgical catheterization. Data are means ± SE. *$P < 0.05$ vs. chow; †$P < 0.05$ vs. wild type.
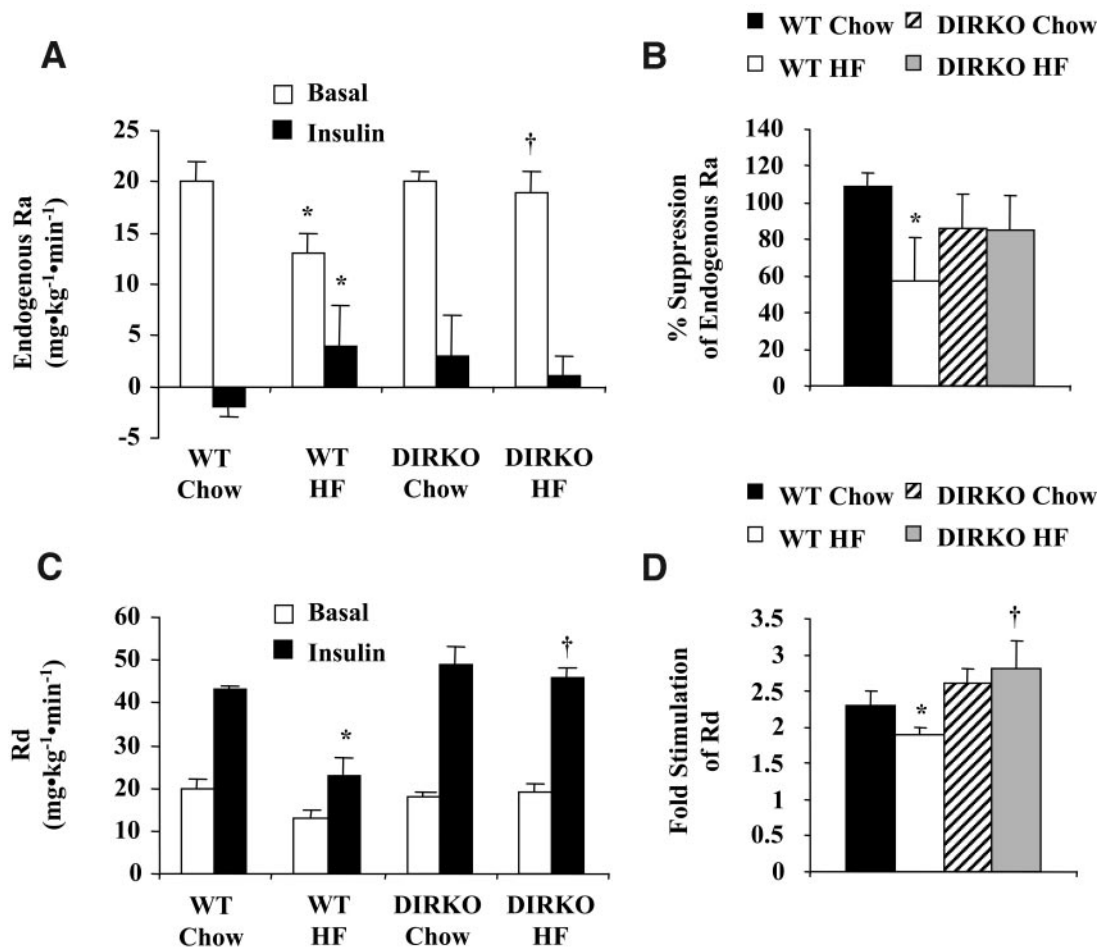
FIG. 5. Whole-body glucose turnover during hyperinsulinemic-euglycemic clamps on 5-h–fasted, conscious, unrestrained chow-fed and high fat–fed C57BL/6 (wild-type) and DIRKO mice. A: Basal and insulin-stimulated endoR_a. B: Insulin-mediated percent suppression of endoR_a. C: Basal and insulin-stimulated R_d. D: Insulin-mediated fold stimulation of R_d. A and C: Basal (white bars) and insulin (black bars). B and D: wild-type chow (black bars), wild-type high fat (white bars), DIRKO chow (striped bars), DIRKO high fat (gray bars). Data are means ± SE for 8–10 mice/group. *$P < 0.05$ vs. chow; †$P < 0.05$ vs. wild type.

cose uptake. Absolute R_g was significantly higher in all muscles analyzed from high fat–fed DIRKO compared with wild-type mice, except for soleus and gastrocnemius (Fig. 6A and B). However, when normalized to clamp insulin levels, R_g was higher in all muscles from high fat–fed DIRKO mice (Fig. 6C and D). The lack of incretin receptors also prevented the high-fat diet–induced decrease in muscle R_g observed in wild-type mice. High-fat feeding did result in decreased heart R_g normalized to clamp insulin levels in DIRKO mice, although not to the degree observed in wild-type mice (Fig. 6D).

DIRKO mice exhibit enhanced liver and muscle insulin signaling. To determine whether the enhanced insulin action in both chow- and high fat–fed DIRKO mice was associated with enhanced insulin signaling, Akt activation, defined as the ratio between phosphorylated (Ser473) Akt and total Akt, was assessed in liver and gastrocnemius muscle. Total gastrocnemius Akt protein levels were not different between wild-type and DIRKO mice on either chow or high-fat diets (Fig. 7A and B). Gastrocnemius Akt activation was significantly higher in DIRKO mice on high-fat diet but not on chow diet (Fig. 7C). Total liver Akt protein was significantly lower in DIRKO mice on chow or high-fat diet compared with wild-type mice (Fig. 7D and E). However, liver Akt activation was significantly en-

hanced in DIRKO mice fed either diet compared with their wild-type counterparts (Fig. 7F).

DISCUSSION

In the present studies, the hyperinsulinemic-euglycemic clamp was used to delineate specific sites where insulin action may be affected by deletion of the receptors for the incretin hormones GLP-1 and GIP. Furthermore, high-fat feeding for 12 weeks was used to precipitate phenotypes associated with the lack of incretin receptor signaling that would otherwise be silent in chow-fed mice. Compared with wild-type mice, DIRKO mice exhibit increased energy expenditure when fed either a chow or a high-fat diet due, at least in part, to increased locomotor activity. Consequently, chow-fed DIRKO mice have reduced weight gain due to decreased muscle mass. High-fat–fed DIRKO mice have reduced weight gain due to decreased fat mass compared with high fat–fed wild-type mice. Here, we show that DIRKO mice exhibit enhanced insulin sensitivity on either chow or high-fat diets. Chow-fed DIRKO mice have decreased insulin levels but demonstrate equal GIR, suppression of endoR_a, and stimulation of R_d during a hyperinsulinemic-euglycemic clamp. Moreover, DIRKO mice are protected from high-fat diet–induced insulin resistance as
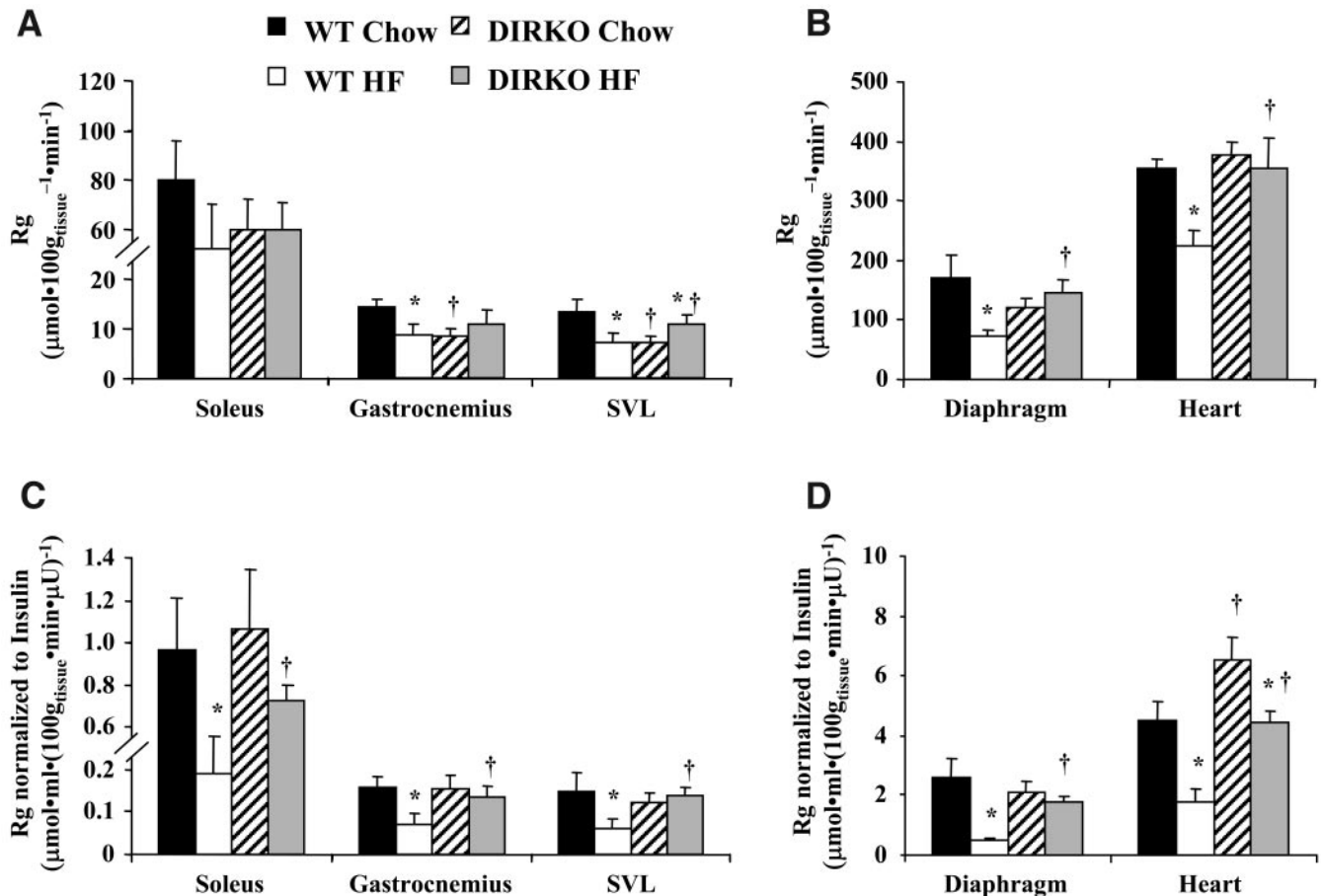
FIG. 6. R_g after hyperinsulinemic-euglycemic clamps on 5-h–fasted, conscious, unrestrained chow-fed and high fat–fed C57BL/6 (wild-type) and DIRKO mice. Wild-type chow (black bars), wild-type high fat (white bars), DIRKO chow (striped bars), and DIRKO high fat (gray bars). *A*: R_g in soleus, gastrocnemius, and SVL. *B*: R_g in diaphragm and heart. *C*: R_g normalized to clamp insulin levels in soleus, gastrocnemius, and SVL. *D*: R_g normalized to clamp insulin levels in diaphragm and heart. Data are means ± SE for 8–10 mice/group. *$P < 0.05$ vs. chow; †$P < 0.05$ vs. wild type.
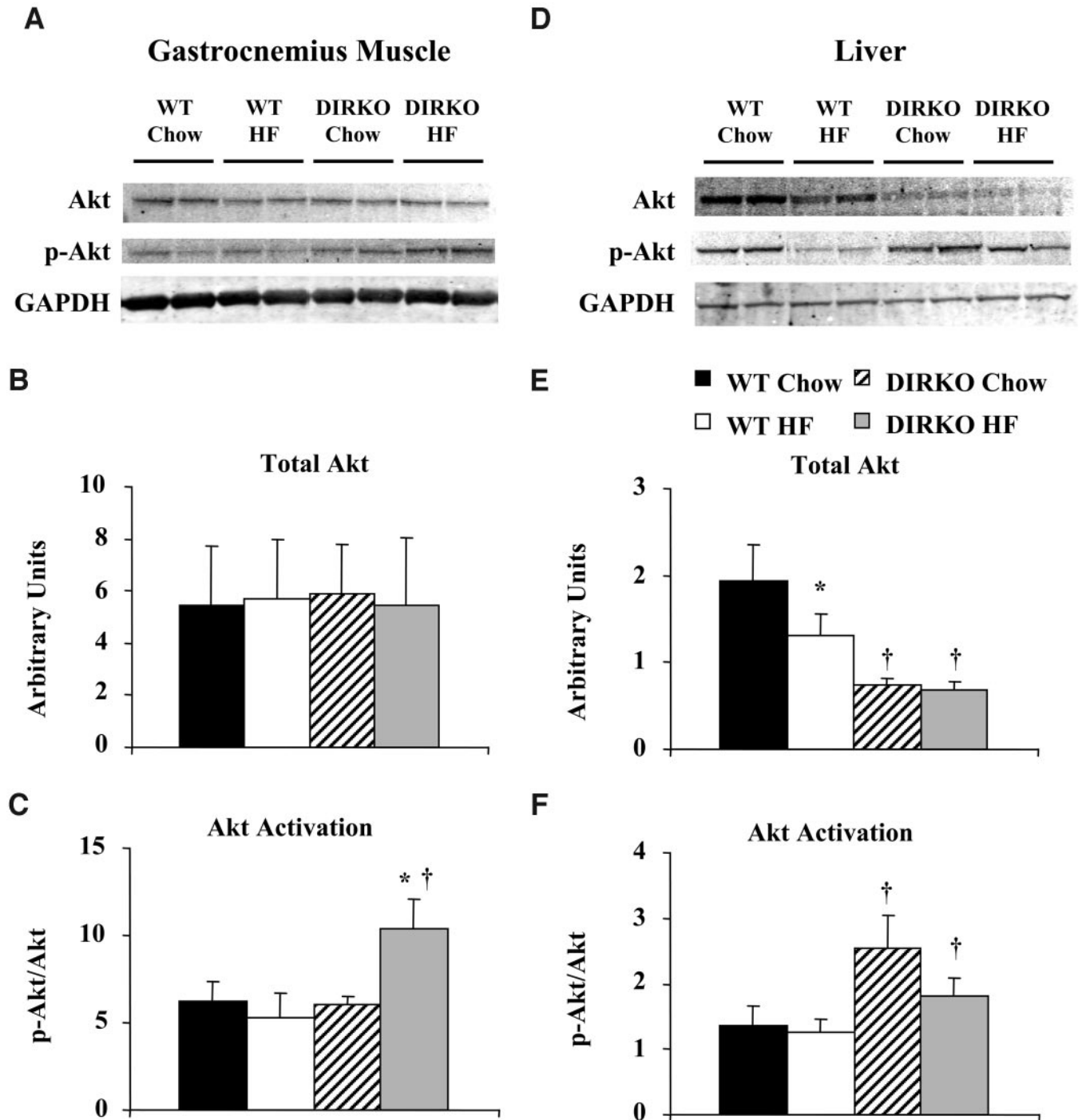
shown by lower fasting glycemia and insulinemia, higher GIR, and suppression of endoR_a, R_d, and R_g during a clamp than high fat–fed wild-type mice. This effect on insulin action was associated with enhanced insulin signaling at both the liver and muscle. Thus, these results demonstrate a role for incretin receptor signaling in the control of insulin action.

A recent study by Hansotia et al. (24) showed increased energy expenditure associated with increased *ucp-1* gene transcription and greater locomotor activity in both DIRKO mice and mice lacking GLP-1R. GLP-1R knockout mice have decreased weight gain on a high-fat diet, although this was only observed in female mice (18). GIPR knockout mice are also protected from high-fat diet–induced obesity due to increased fat oxidation and energy expenditure (13). This was also observed in a model of genetic obesity, the *ob/ob* mouse, crossed with GIPR knockout mice (13). In the present studies, increased energy expenditure in both chow- and high fat–fed DIRKO mice was associated with increased locomotor activity, but the lack of an effect on the RER suggests that fat oxidation was not increased in DIRKO mice. However, NEFA levels were lower in DIRKO mice on either chow or high-fat diets, even as insulin levels were also lower. Thus, an effect of disrupted incretin signaling on increased fat oxidation cannot be ruled out. Fat absorption was also not impaired in high fat–fed DIRKO mice, because the percent

of ingested fat excreted in the feces was not different compared with wild-type mice.

The results obtained from the present studies are interesting in that a mutation that impairs β-cell function also preserves insulin sensitivity. This is especially evident in the presence of a metabolic stressor, such as high-fat feeding. Wild-type mice respond to chronic high-fat feeding by significantly increasing β-cell area, islet number, and pancreatic insulin content and are more insulin resistant. Hansotia et al. (24) showed that, in response to chronic high-fat feeding, β-cell area and islet number are increased in DIRKO mice, although not to the degree observed in wild-type mice. Unlike wild-type mice on a high-fat diet, pancreatic insulin content in high fat–fed DIRKO mice is not increased (24), resulting in significantly lower fasting insulin levels, as shown in the present studies. However, this impaired morphological and functional pancreatic response to high-fat feeding in DIRKO mice is offset by preservation of hepatic and peripheral insulin sensitivity.

Obesity is a significant risk factor associated with the development of insulin resistance and type 2 diabetes (32,33). Thus, decreased obesity is likely associated with protection from high-fat diet–induced insulin resistance in DIRKO mice. Consistent with this, chemical ablation of GIPR with (Pro[3])GIP, a GIPR antagonist, has been shown to decrease adiposity, improve glucose tolerance, and

FIG. 7. Immunoblots after hyperinsulinemic-euglycemic clamps on 5-h–fasted, conscious, unrestrained chow-fed and high fat–fed C57BL/6 (wild-type) and DIRKO mice. Wild-type chow (black bars), wild-type high fat (white bars), DIRKO chow (striped bars), DIRKO high fat (gray bars). _A_: Representative images of immunoblots from gastrocnemius extracts for total Akt (Akt), phosphorylated Akt (_p_-Akt), and GAPDH. _B_: Total Akt normalized to GAPDH in gastrocnemius. _C_: Akt activation (_p_-Akt/total Akt) in gastrocnemius. _D_: Representative images of immunoblots from liver extracts for total Akt, _p_-Akt, and GAPDH. _E_: Akt normalized to GAPDH in liver. _F_: Akt activation in liver. Data are means ± SE for 8–10 mice/group. *$P < 0.05$ vs. chow; †$P < 0.05$ vs. wild type.

modestly enhance the response to intraperitoneal insulin in _ob/ob_ mice (34). Similarly, tissue lipid accumulation is reduced and the glucose response to an intraperitoneal insulin injection is preserved in high fat–fed GLP-1R knockout and DIRKO mice compared with high fat–fed wild-type mice (24). To directly address the effect of a disruption in incretin signaling on insulin action, the present studies used the hyperinsulinemic-euglycemic clamp in conscious, unrestrained mice. This technique is

generally considered the "gold standard" for assessment of in vivo insulin action. The addition of isotopic glucose tracers with this technique allows for the determination of specific sites where insulin action is affected. Whereas high-fat feeding impairs insulin-mediated suppression of endoR$_a$ and stimulation of R_d in wild-type mice, these impairments do not occur in high fat–fed DIRKO mice. DIRKO mice on a high-fat diet have a more than threefold higher index of insulin sensitivity (GIR/[insulin]) than high

fat–fed wild-type mice. Furthermore, R_g is impaired in wild-type mice fed a high-fat diet, whereas DIRKO mice do not exhibit such a diet-induced impairment. This protection from high fat diet–induced insulin resistance in DIRKO mice is associated with enhanced insulin signaling, because hepatic and muscle Akt activation is greater in high fat–fed DIRKO mice compared with their wild-type counterparts.

Recent evidence indicates that GLP-1 action in the brain can acutely regulate peripheral insulin sensitivity. Knauf et al. (35) showed that glucose requirements during a hyperinsulinemic-hyperglycemic clamp are higher in mice receiving an intracerebroventricular infusion of the GLP-1R antagonist exendin 9-39. Conversely, mice receiving the GLP-1R agonist exendin 4 directly into the brain showed decreased glucose requirements. 2-Deoxyglucose uptake into skeletal muscle was increased in mice receiving intracerebroventricular exendin 9-39 and decreased in mice receiving exendin 4 under hyperinsulinemic-hyperglycemic conditions. These studies suggest that endogenous GLP-1 action in the brain inhibits muscle insulin action. Thus, in agreement with the present studies, the disruption of central GLP-1 action enhances peripheral insulin sensitivity.

Phenotypes associated with insulin action were also observed in chow-fed DIRKO mice. Absolute R_g in three different skeletal muscles was lower in DIRKO than wild-type mice. However, when normalized to clamp insulin levels, there was no difference in muscle R_g between these two groups. Interestingly, Akt protein expression in the gastrocnemius was lower in DIRKO mice, but Akt activation was higher. This was evident even as clamp insulin levels were lower in DIRKO mice. R_g in the heart was also higher in DIRKO compared with wild-type mice. This is likely due to loss of GLP-1R signaling, because this incretin receptor, and not GIPR, is expressed in the heart (36,37).

DIRKO mice exhibited lower fasting NEFA levels on both chow and high-fat diets, even as fasting insulin levels were insignificantly lower in chow-fed mice and significantly lower in high fat–fed mice. Activation of GIPR in cultured 3T3-L1 adipocytes stimulates heparin-releasable lipoprotein lipase activity, increasing NEFA levels (13). Thus, disruption of GIPR signaling in adipocytes likely results in decreased fasting NEFA levels in DIRKO mice. Although insulin-mediated suppression of NEFA levels was equal in wild-type and DIRKO mice, we cannot rule out a direct effect of disrupting incretin signaling on lipid turnover.

Taken together, these studies demonstrate that although complete disruption of incretin receptor signaling results in impaired β-cell function and adaptation to chronic high-fat feeding (24), this is overcome by preservation of insulin action. This is likely secondary to effects on energy expenditure, activity, and adiposity associated with the lack of incretin receptor signaling. Although disruption of GIPR results in impaired β-cell function, it also results in increased energy expenditure and decreased adiposity. Similarly, the deleterious effects of ablating GLP-1R signaling on β-cell function are overcome by increased activity-dependent energy expenditure. Thus, the absence of incretin receptor signaling results in a metabolic status capable of responding to impaired β-cell function and the stress of high-fat feeding. These results extend the importance of incretin action in regulating glucose homeostasis beyond the pancreas and demonstrate how incretin receptor signaling can modulate insulin action by mechanisms other than stimulation of insulin secretion.

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