Improved Insulin Sensitivity and Resistance to Weight Gain in Mice Null for the *Ahsg* Gene

Suresh T. Mathews,[1,2] Gurmant P. Singh,[1] Mollie Ranalletta,[3] Vivian J. Cintron,[2] Xiaoling Qiang,[2] Anton Scott Goustin,[2] Kai-Lin Catherine Jen,[4] Maureen J. Charron,[3] Willi Jahnen-Dechent,[5] and George Grunberger[1,2]

Fetuin inhibits insulin-induced insulin receptor (IR) autophosphorylation and tyrosine kinase activity in vitro, in intact cells, and in vivo. The fetuin gene (*AHSG*) is located on human chromosome 3q27, recently identified as a susceptibility locus for type 2 diabetes and the metabolic syndrome. Here, we explore insulin signaling, glucose homeostasis, and the effect of a high-fat diet on weight gain, body fat composition, and glucose disposal in mice carrying two null alleles for the gene encoding fetuin, *Ahsg* (B6, 129-Ahsg^{tm1Mbl}). Fetuin knockout (KO) mice demonstrate increased basal and insulin-stimulated phosphorylation of IR and the downstream signaling molecules mitogen-activated protein kinase (MAPK) and Akt in liver and skeletal muscle. Glucose and insulin tolerance tests in fetuin KO mice indicate significantly enhanced glucose clearance and insulin sensitivity. Fetuin KO mice subjected to euglycemic-hyperinsulinemic clamp show augmented sensitivity to insulin, evidenced by increased glucose infusion rate ($P = 0.077$) and significantly increased skeletal muscle glycogen content ($P < 0.05$). When fed a high-fat diet, fetuin KO mice are resistant to weight gain, demonstrate significantly decreased body fat, and remain insulin sensitive. These data suggest that fetuin may play a significant role in regulating postprandial glucose disposal, insulin sensitivity, weight gain, and fat accumulation and may be a novel therapeutic target in the treatment of type 2 diabetes, obesity, and other insulin-resistant conditions. *Diabetes* 51:2450–2458, 2002

From the [1]Department of Internal Medicine, Wayne State University School of Medicine, Detroit, Michigan; the [2]Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, Michigan; the [3]Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York; the [4]Nutrition and Food Science Department, Wayne State University, Detroit, Michigan; and [5]IZKF BIOMAT, Klinikum der RWTH Aachen, Aachen, Germany.

Address correspondence and reprint requests to George Grunberger, Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, University Health Center, 4H, 4201 St. Antoine Blvd., Detroit, MI 48201. E-mail: g.grunberger@wayne.edu.

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2-DOG, 2-deoxyglucose; α_2-HSG, α_2-HS-glycoprotein; AIN, American Institute of Nutrition; BMR, basal metabolic rate; ERK2, extracellular signal–regulated kinase 2; FFA, free fatty acid; GIR, glucose infusion rate; GTT, glucose tolerance test; HF, high-fat; HOMA, homeostasis model assessment; IR, insulin receptor; IRS, insulin receptor substrate; ITT, insulin tolerance test; KO, knockout; LF, low-fat; MAPK, mitogen-activated protein kinase; PPAR-γ, peroxisome proliferator–activated receptor-γ; PTP1B, protein tyrosine phosphatase 1B; $S_{0.5}$, half-saturating substrate concentration; TG, triglyceride; TK, tyrosine kinase; WGA, wheat germ agglutinin; WT, wild type.

Worldwide prevalence data indicate that type 2 diabetes has reached epidemic proportions (1). Parallel to the rise in type 2 diabetes is a rapid expansion of obesity, associated with consumption of a high-fat diet (2). Insulin resistance is central to the pathophysiology of obesity and type 2 diabetes (3). Several physiological modulators of insulin receptor (IR) function involved in the pathogenesis of insulin resistance have been described, namely tumor necrosis factor-α, PC-1 (plasma cell 1), Rad, protein tyrosine phosphatases, and α_2-HS-glycoprotein (α_2-HSG)/fetuin (4–8).

Human α_2-HSG/fetuin is a natural inhibitor of the insulin-stimulated IR tyrosine kinase (TK) (8–13). Phosphorylation status of α_2-HSG and rat fetuin is of critical importance for TK inhibition (9,14). Nearly 20% of the circulating α_2-HSG pool is phosphorylated on Ser-120 and Ser-312 to ~1 mole phosphate per mole protein (15). α_2-HSG inhibits IRTK by reducing the V_{max} of the insulin-stimulated IRTK reaction and increasing the half-saturating substrate concentration ($S_{0.5}$) for ATP and the exogenous substrate poly(Glu^{80} Tyr^{20}) (16). α_2-HSG preferentially interacts with the activated IR and does not require the proximal 576 amino acids of the IR α-subunit for its IR autophosphorylation/TK inhibitory activity (17). Acute injection of human recombinant α_2-HSG inhibits insulin-stimulated tyrosine phosphorylation of IR β-subunit and insulin receptor substrate (IRS)-1 in rat liver and skeletal muscle (17). In a rat model of diet-induced obesity, a significant increase in fetuin gene expression has been observed (18). Evidence of IRTK inhibitory function of human α_2-HSG and bovine, mouse, sheep, and pig fetuin suggests a conserved function for fetuin homologs (8,16,19,20). The human *AHSG* gene resides on chromosome 3q27, which recently has been mapped as a type 2 diabetes susceptibility locus (21). Kissebah et al. (22) have demonstrated a quantitative trait locus on chromosome 3q27 strongly linked to the metabolic syndrome. Mice with a targeted deletion of the fetuin gene (*Ahsg*) are fertile and demonstrate no gross anatomical abnormalities except for the presence of ectopic microcalcifications in a minority of retired female breeders (23). In humans, no complete α_2-HSG deficiency has been found in extensive population studies and clinical investigations (24).

To clarify the role of fetuin in insulin action, we explored glucose homeostasis in mice carrying two null

alleles for *Ahsg*. Because fetuin inhibits insulin-induced IR autophosphorylation and TK activity, it is hypothesized that genetic ablation of fetuin results in enhanced insulin signal transduction and increased whole-body insulin sensitivity. Further, the consequence of this genetic manipulation is examined in a model of acquired insulin resistance, high-fat feeding.

RESEARCH DESIGN AND METHODS

Animals. Homozygous *Ahsg* knockout (*Ahsg*$^{-/-}$) mice (23) from a mixed background were backcrossed four generations into C57Bl/6J. Offspring (*Ahsg* knockout [KO] and wild-type [WT] littermates [*Ahsg*$^{+/+}$]) from the fourth generation of the breeding protocol were used for this study. Mice were housed on a 12-h light/dark cycle and fed a standard rodent food. All protocols for animal use and euthanasia were reviewed and approved by the Animal Investigation Committee of Wayne State University in accordance with National Institutes of Health guidelines. For in vivo studies, animals were anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg) intraperitoneally (i.p.), and insulin (0.1, 1, and 10 μmol/l) was injected through the portal vein. Saline-injected animals served as controls. Liver and hindlimb muscles were excised 1 and 3 min later, respectively, as described earlier (25).

Surgical procedures. Mice were anesthetized with an injection of pentobarbital (65 mg/g body wt i.p.), and an indwelling catheter was implanted as described earlier (26). During a 2-day observation period, mice exhibiting signs of illness were excluded from the study (two animals).

High-fat feeding. Mice (n = 43) were housed four to five per hanging cage with food and water available ad libitum. Within each genotype (KO and WT), they were divided into high-fat (HF) and low-fat (LF) groups. The LF diet was based on the American Institute of Nutrition (AIN)-93 mol/l formula (27) with 4% fat in the form of soybean oil. The HF diet was a modification of the AIN-93 mol/l formula with added soybean oil so that the final fat content was 40% by weight. Carbohydrate, protein, and fat contents as a percentage of caloric content were 75.9%, 14.1%, and 10% for LF and 26.17%, 15.06%, and 58.77% for HF (Dyets, Bethlehem, PA). WT and KO mice were fed HF or LF diets for a period of 9 weeks. Food intake and body weight were measured once a week. For body composition analysis, internal organs were dissected and all visible internal fat was removed and weighed. The remaining carcass was frozen for carcass analysis (28). The carcass fat, designated subcutaneous fat, was extracted by the method of Folch et al. (29). Total body fat was calculated as the sum of subcutaneous fat and internal fat.

Partial purification of IR autophosphorylation and TK activity. IRs were partially purified on wheat germ agglutinin (WGA)-agarose columns and eluted with 0.3 mol/l *N*-acetylglucosamine. IR autophosphorylation of the partially purified IR, in the presence or absence of insulin, was carried out by the addition of [γ-^{32}P]ATP to a reaction mixture containing 5 mmol/l $MnCl_2$, 50 μmol/l ATP, 50 mmol/l HEPES, pH 7.6, and 0.1% Triton X-100, and the proteins were then separated on 7.5% SDS-PAGE. IRTK activity was assayed by quantitation of phosphorylation on exogenous substrate, poly(Glu^{80}Tyr^{20}) (17).

Metabolic studies. For glucose tolerance tests (GTTs), an oral (1 mg/g) or i.p. (1.5 mg/g) glucose load was given after a 16-h fast to 10-week old male or female WT and fetuin KO mice. Blood samples were taken at 0, 15, 30, 60, and 120 min from the tail vein. Glucose levels were measured with a Glucometer Elite monitor (Bayer, Elkhart, IN). For insulin tolerance tests (ITTs), random-fed female mice, all 10 weeks of age, were given an i.p. injection of 0.75 or 0.15 U/kg regular human insulin (Novo Nordisk, Clayton, NC) between 2:00 and 5:00 P.M. Blood samples were obtained at various time points from the tail vein, and glucose levels were measured as described above. Insulin levels were measured in plasma using rat insulin standards (Linco Research, St. Charles, MO). To assess lipid levels, blood samples were obtained by retro-orbital bleeds from overnight-fasted anesthetized mice. Fasting triglyceride (TG) levels were measured in plasma by a colorimetric assay (Sigma), and fasting free fatty acid (FFA) concentrations were determined using the NEFA C kit (Wako Chemicals, Richmond, VA). Fasting levels of leptin were assayed with a mouse leptin radioimmunoassay kit (Linco Research).

Euglycemic-hyperinsulinemic clamp. Clamp studies were carried out on five male KO mice (3–4 months old) and five age- and sex-matched WT mice (26,30). Food was removed 5–6 h before infusion. A bolus of [3-^{3}H]glucose (50 μCi) was administered at the start of each clamp over 1 min. For the remainder of the clamp, [3-^{3}H]glucose was infused at 12 μCi · kg^{-1} · min^{-1}. A continuous infusion of porcine insulin (Eli Lilly, Indianapolis, IN) was administered at 100 mU · kg^{-1} · min^{-1}. Plasma glucose was clamped at 90–110 mg/dl by infusing a 20% glucose solution. Glycemia was assessed on blood obtained from the tail vein using a One Touch II Meter (LifeScan, Milpita, CA). Steady-state glucose levels were achieved after ~80 min, at which point 10 μl blood was collected every 10 min for 40 min. The animals were then given a bolus (24 μCi) of [^{14}C]-labeled 2-deoxyglucose (2-DOG), which was flash-injected through the catheter, and 10 μl blood was collected at 2, 4, 6, 8, 10, 20, 30, and 40 min. At the end of the 40-min period, the animals were killed. Tissues (brown and white adipose, heart, diaphragm, soleus, extensor digitorum longus, gastrocnemius, and skin) were rapidly removed and snap-frozen in liquid nitrogen for further analysis. Whole-body glucose utilization and tissue 2-DOG uptake were calculated as previously described (26,30). Muscle glycogen content was determined by the amyloglucosidase method (31).

Antibodies. Antibodies against IR β-subunit, phosphotyrosine proteins (4G10), and extracellular signal–regulated kinase 2 (ERK2) were purchased from Upstate Biotechnology (Lake Placid, NY). p44/42 mitogen-activated protein kinase (MAPK) assay kit, phospho-p44/42 MAPK antibody, and phospho-Akt antibody were purchased from New England Biolabs (Beverly, MA).

Immunoprecipitations and immunoblotting. Liver and muscle tissues were excised and homogenized in ice-cold buffer A (50 mmol/l HEPES, pH 7.4, 25 mmol/l NaPPi, 100 mmol/l NaF, 10 mmol/l EDTA, 10 mmol/l Na_3VO_4, 2 mmol/l phenylmethylsulfonyl fluoride, 1% Triton X-100, and 10 μg/ml aprotinin and leupeptin). Immunoprecipitations were carried out overnight at 4°C with required antibodies followed by addition of protein A and G sepharose beads (Oncogene, Cambridge, MA) for another hour at 4°C. Immunoprecipitated proteins (IR β-subunit, phosphorylated p44/42 MAPK) were washed, boiled in SDS-sample buffer, separated on 7.5% SDS-PAGE, transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, NH), and developed using appropriate combinations of primary/secondary antibodies and chemiluminescence. Phosphorylation status of MAPK and Akt was assayed by Western blotting using phospho-p44/42 MAPK antibody and phospho-Akt antibody, respectively. Quantitation of ERK2, IR-β subunit, and Akt-1 were done to normalize the phosphorylation data to protein loading. MAPK activity (phosphorylation of Elk-1) was assayed using a kit with two phospho-specific antibodies (New England Biolabs).

Statistical Analysis. Data are presented as means ± SE. Statistical analyses (Student's t test or ANOVA) were performed using GraphPad Instat (San Diego, CA). Differences were considered significant if $P \leq 0.05$. Quantitation of data from Western blots and autoradiographs were done using UN-SCAN-IT Gel Digitizing System (Silk Scientific, Orem, UT).

RESULTS

Increased IR autophosphorylation and tyrosine kinase activity. Because α_2-HSG/fetuin inhibits insulin-induced IR autophosphorylation and TK activity, we examined the consequence of genetic ablation of fetuin on basal and insulin-induced IR autophosphorylation status in vitro (partially purified IR) and in vivo (liver and skeletal muscle). IR purified from livers of fetuin KO mice showed an approximate fourfold increase in basal IR autophosphorylation in vitro, compared with IR from livers of WT mice (Fig. 1*A*, upper panel). Insulin (1 nmol/l) induced a ~2-fold increase in IR β-subunit phosphorylation in KO mice (14.26 ± 1.55-fold stimulation over WT basal [arbitrary scan units]) (Fig. 1*A*, lower panel) compared with WT mice (8.56 ± 1.38 arbitrary scan units). Insulin-induced IR autophosphorylation was similar at higher insulin concentrations (10 or 100 nmol/l) in WT and KO mice. Western blotting with an antibody against IR β-subunit confirmed equal amounts of IR loading in both WT and KO lanes (Fig. 1*A*, lower panel). TK activity was assayed in vitro in WGA-purified IR from KO and WT mice. Basal TK activity was significantly increased ($P < 0.001$) in IR from KO mice (Fig. 1*B*), analogous to results of receptor autophosphorylation. Next, autophosphorylation of IR β-subunit in liver and skeletal muscle, after in vivo exposure to insulin (portal vein injection of 0.1, 1, or 10 μmol/l insulin), was assayed in age-, weight-, and sex-matched WT and KO mice. Saline-injected mice served as controls. Figure 1 shows representative blots of IR autophosphorylation in liver (*C*, upper panel) and skeletal

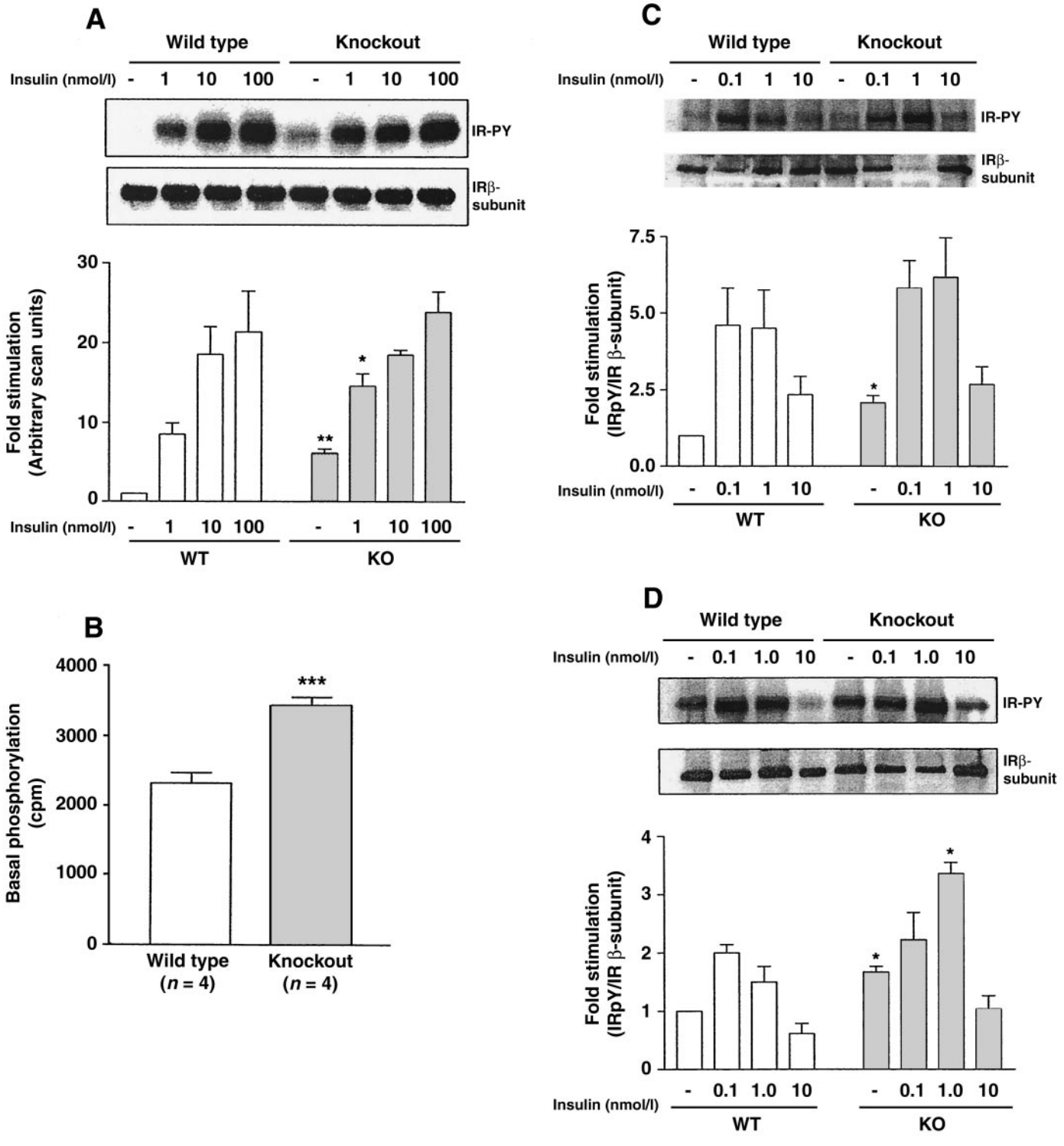

FIG. 1. IR autophosphorylation and TK activity. In vitro IR autophosphorylation (*A*) and IRTK activity (*B*) were determined from IRs partially purified from liver membrane fraction on WGA. *A*: A representative autoradiograph of in vitro IR β-subunit autophosphorylation (PY) (basal or in the presence of 1, 10, or 100 nmol/l insulin) (upper panel). Four separate experiments were performed, with IRs purified individually from livers of weight-matched, 8- to 10-week-old male WT and KO mice; $n = 4$ per genotype. Western blot of IR β-subunit confirmed equal loading of IR (lower panel), and the result of the combined data of four separate experiments is shown (bar diagram). To assess the status of in vivo basal and insulin-induced IR autophosphorylation, saline or insulin (0.1, 1, or 10 μmol/l) was injected through the portal vein of weight-matched, 8- to 10-week-old male WT and KO mice. IR was immunoprecipitated from liver (*C*) or muscle (*D*) homogenates with anti–IR β-subunit antibody and immunoblotted with anti-phosphotyrosine antibody. Samples were normalized for loading by assaying total level of IR β-subunit. The quantified data (ratio of IR autophosphorylation to total level of IR β-subunit) are shown as bar diagrams (mean ± SE; $n = 4$ mice per genotype). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, WT vs. KO.

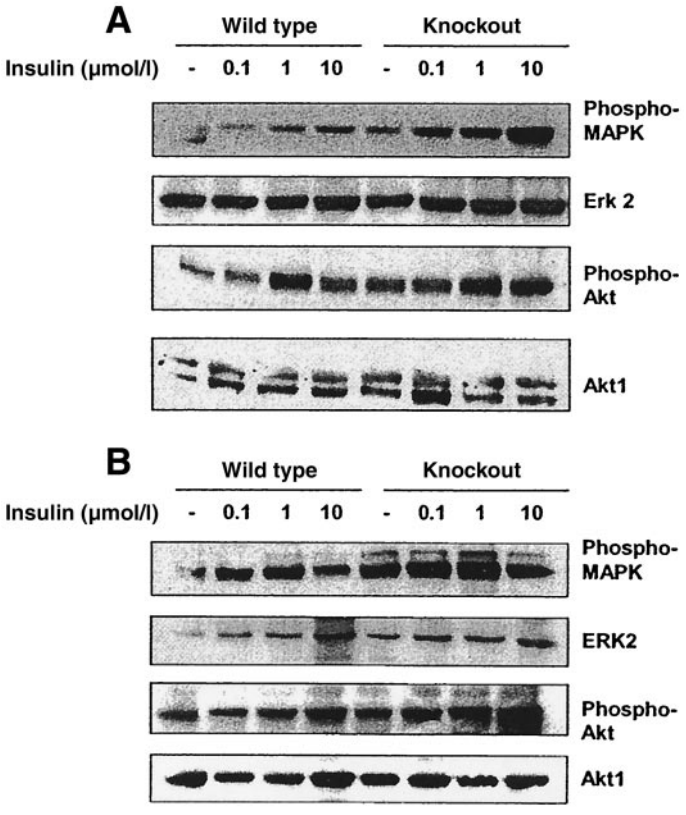

FIG. 2. Insulin signal transduction. *A*: Liver homogenates from saline- or insulin-injected (0.1, 1, or 10 μmol/l) weight-matched, 8- to 10-week-old male WT and KO mice were resolved on SDS-PAGE, transferred, and detected by chemiluminescence with antibodies against phospho-MAPK or phospho-Akt. Membranes were stripped and blotted for ERK2 and Akt1, respectively, to normalize for sample loading. A representative blot (from four to five separate experiments) for each protein is depicted. *B*: Hindlimb muscle homogenates were resolved on SDS-PAGE, transferred, and detected by chemiluminescence with antibodies against phospho-MAPK or phospho-Akt. Membranes were stripped and blotted for ERK2 and Akt1, respectively, to normalize for sample loading. A representative blot (from four to five separate experiments) for each protein is depicted.

muscle (*D*, upper panel). IR phosphorylation data in liver and muscle were normalized to IR β-subunit levels (Fig. 1*C*, lower panel, and *D*, lower panel), and the combined data from four separate experiments are shown as bar diagrams. A 2-fold increase in basal IR autophosphorylation in liver ($P < 0.05$) and a ~1.5-fold increase ($P < 0.05$) in basal IR phosphorylation in skeletal muscle were observed in fetuin KO mice. A significant increase ($P < 0.05$) in insulin-induced (1 μmol/l) IR phosphorylation was observed in skeletal muscle of KO mice, and a similar increasing trend in insulin-induced IR phosphorylation was observed in livers from KO mice.

Increased MAPK and Akt phosphorylation. To confirm that the increased phosphorylation of IR in liver and muscle of fetuin KO mice is manifested in increased downstream signaling, phosphorylation status of p44/42 MAPK and Akt were assayed after in vivo exposure to insulin (portal vein injection of 0.1, 1, or 10 μmol/l insulin) or saline in age-, weight-, and sex-matched WT and KO mice. All assays were done in four to five mice in each group; Fig. 2 shows a representative blot for each protein.

In liver, phosphorylation of MAPK was assayed by phospho-p44/42 MAPK antibody and its activity by detecting MAPK-induced phosphorylation of Elk-1. In livers of KO mice, basal phosphorylation of p44/42 MAP kinase was significantly increased (phospho-MAPK/ERK2 ratio for KO, 1.54 ± 0.11-fold stimulation over WT; $P < 0.05$) (Fig. 2*A*). Injection of insulin through the portal vein induced a significant increase in p44/42 MAPK phosphorylation compared with WT mice for every dose of insulin tested (phospho-MAPK/ERK2 ratio with 10 μmol/l insulin for KO, 3.78 ± 0.24-fold stimulation over WT basal; for WT, 2.23 ± 0.24-fold; $P < 0.001$) (Fig. 2*A*). Reprobing the membrane with ERK2 antibody confirmed equal sample loading (Fig. 2*A*). MAPK activity assayed in liver homogenates (by active MAPK phosphorylation of Elk-1) demonstrated increased basal and insulin-stimulated phosphorylation of phospho-Elk-1, in concurrence with p44/42 MAPK phosphorylation (data not shown). Similarly, both basal (phospho-Akt/Akt1 ratio for KO, 1.45 ± 0.15-fold stimulation over WT; $P < 0.05$) and insulin-induced (phospho-Akt/Akt1 ratio with 10 μmol/l insulin for KO, 2.2 ± 0.09-fold stimulation over WT basal; for WT, 1.57 ± 0.09-fold; $P < 0.01$) phosphorylation of Akt, measured by phospho-Akt antibody, was significantly increased in liver homogenates of fetuin KO mice compared with WT mice (Fig. 2*A*). Equal loading was confirmed using an antibody against Akt-1 (Fig. 2*A*).

In skeletal muscle, all tested doses of insulin induced greater amounts of p44/42 MAPK phosphorylation in fetuin KO mice compared with WT mice (phospho-MAPK/ERK2 ratio with 10 μmol/l insulin for KO, 5.73 ± 0.34-fold stimulation over WT basal; for WT, 3.23 ± 0.29-fold; $P < 0.01$) (Fig. 2*B*). Equal loading of all lanes was confirmed using an antibody against ERK2 (Fig. 2*B*). Insulin-induced phosphorylation of Akt, measured by phospho-Akt antibody, was also increased in muscle (phospho-Akt/Akt1 ratio with 10 μmol/l insulin for KO, 3.00 ± 1.19-fold stimulation over WT basal; for WT, 1.63 ± 0.12-fold; $P < 0.001$) (Fig. 2*B*) of fetuin KO mice compared with WT mice. Equal loading was confirmed by similar concentrations of Akt-1 (Fig. 2*B*). Basal phosphorylation of both MAPK (KO, 2.65 ± 0.38-fold stimulation over WT; $P < 0.01$) and Akt (KO, 1.38 ± 0.04-fold stimulation over WT; $P < 0.01$) was increased in skeletal muscle of KO mice. A representative blot from four to five separate experiments for each protein is depicted in Fig. 2*B*.

Enhanced glucose clearance and increased insulin sensitivity in fetuin KO mice. Because fetuin KO mice demonstrated increased insulin signaling, we examined glucose clearance rates using GTTs and ITTs in 8- to 10-week-old KO and WT mice. Fetuin KO mice cleared postprandial glucose from the blood with an increased efficiency over WT mice during oral GTTs (1 mg/g). Differences between WT and KO blood glucose values were statistically significant ($P < 0.01$) at the 15-, 30-, and 60-min time points (Fig. 3*A*). This experiment was repeated twice using different sets and sexes of animals ($n = 6$/group) with similar results (data not shown). Although fetuin KO mice, on average, have significantly lower body weights ($P = 0.005$) than age- and sex-matched controls (10-week-old female WT, 15.5 ± 1.2 g, $n = 14$; 10-week-old female KO, 12.2 ± 0.9 g, $n = 17$; $P < 0.001$) (Table 1), the body weights for KO and WT mice undergoing the oral GTT were not statistically different (WT, 17.54 ± 2.21 g;

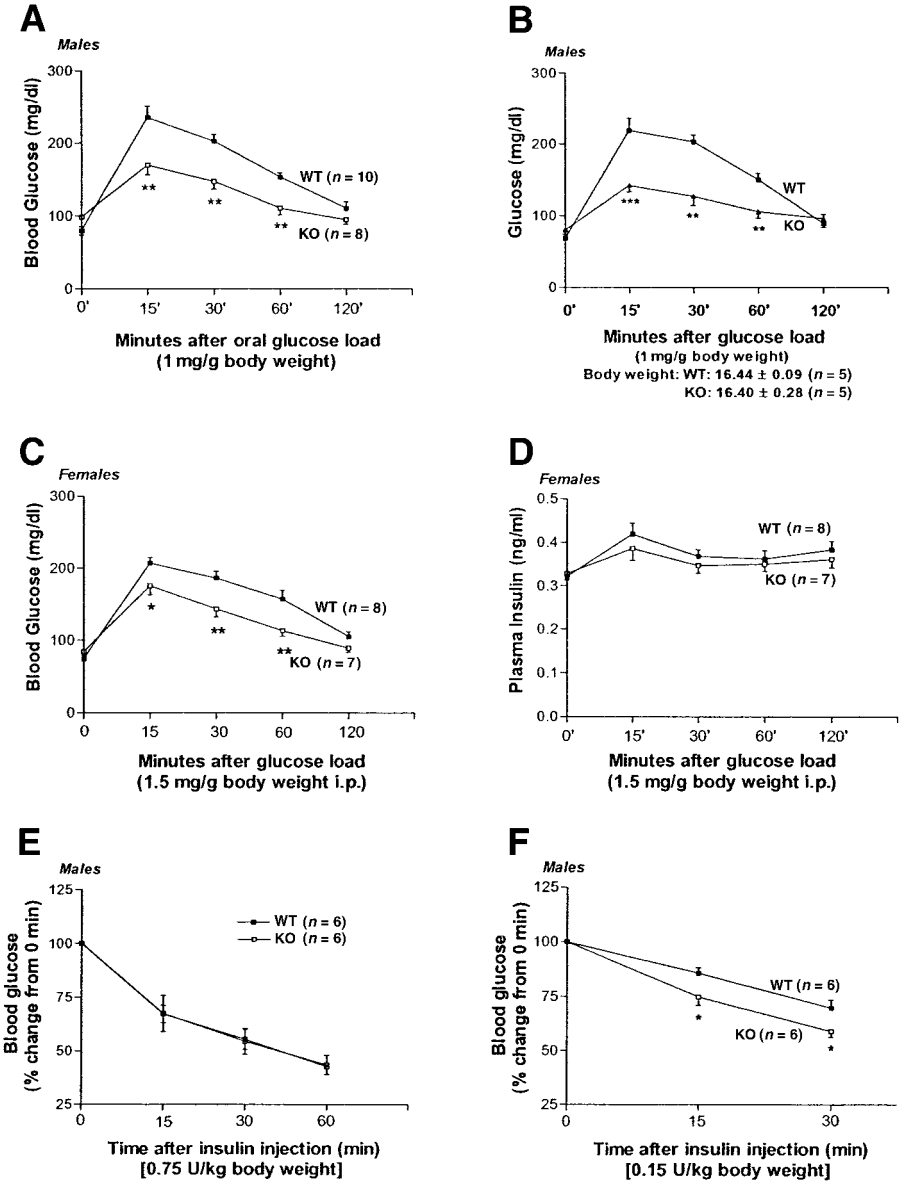

FIG. 3. Glucose and insulin tolerance tests in KO and WT mice. After an overnight fast, an oral glucose load (1 mg/g) (*A* and *B*) or i.p. glucose load (1.5 mg/g) (*C* and *D*) was given to 10-week-old fetuin KO and WT mice. Insulin tolerance tests were done on fed (random) mice using an i.p. injection of 0.75 units/kg (*E*) or 0.15 units/kg (*F*) regular human insulin. Blood glucose (*A*, *B*, *C*, *E*, *F*) or plasma insulin (*D*) was measured as described in RESEARCH DESIGN AND METHODS. Results shown are from either male or female mice, as similar findings were observed in both male and female mice. Data are means ± SE. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, WT vs. KO.

KO, 15.84 ± 1.51 g; $P = 0.06$) since the body weights of some WT and KO mice overlap. However, to exclude the possibility of body weight affecting the outcome of the test, oral GTTs were also done on age-, sex-, and weight-matched mice (WT, 16.44 ± 0.09 g, $n = 5$; KO, 16.40 ± 0.28 g, $n = 5$) with similar results (Fig. 3*B*). Further, GTTs

TABLE 1
Body weight, blood glucose, and plasma measurements in 10-week-old KO and WT mice

	Wild type	Knockout	*P*
Body weight (g)	17.9 ± 0.8 (11)	13.9 ± 0.9 (10)	<0.01
Fasting blood glucose (mg/dl)	79.8 ± 5.9 (9)	98.3 ± 7.7 (10)	0.245
Fed blood glucose (mg/dl)	120.2 ± 6.4 (11)	107.0 ± 10.0 (10)	0.272
Fasting insulin (ng/ml)	0.177 ± 0.01 (11)	0.191 ± 0.01 (10)	0.337
Fed insulin (ng/ml)	0.213 ± 0.02 (7)	0.187 ± 0.02 (8)	0.448
Fasting FFAs (mEq/l)	0.827 ± 0.06 (11)	0.599 ± .03 (11)	<0.001
Fasting triglycerides (mg/dl)	59.52 ± 1.71 (12)	42.31 ± 4.33 (11)	<0.001
Fasting leptin (ng/ml)	1.33 ± 0.07 (12)	1.63 ± 0.46 (12)	0.068

Data are means ± SE (number of animals). Body weight, glucose, and insulin were measured in male mice; FFAs, triglycerides, and leptin were measured in female mice.

were also done with i.p. injections of glucose (1.5 mg/g; mean WT body weight, 22.26 g; KO, 22.77 g) to exclude the possibility of defective gastrointestinal absorption or absorption-related mechanisms. As in oral GTTs, diverging curves were obtained for the i.p. GTTs, with KO mice displaying significantly enhanced glucose disposal compared with WT mice ($P < 0.05$ for 15 min; $P < 0.01$ for 30 and 60 min) (Fig. 3*C*). Plasma insulin concentrations after an i.p. (Fig. 3*D*) or oral (data not shown) glucose load demonstrated identical responses in KO and WT mice. To confirm enhanced insulin sensitivity, ITTs were done on 8- to 10-week-old male WT and KO animals fed ad libitum. Using a single i.p. injection of regular human insulin (Novolin) (0.75 units/kg), no differences were observed in the clearance of glucose from the blood of WT and KO mice (Fig. 3*E*). However, when a lower dose of insulin was injected (0.15 units/kg), there was a ~45% drop in blood glucose in KO mice at 30 min, versus ~30% in WT ($P < 0.05$) (Fig. 3*F*).

Fasting or fed (random) blood glucose or plasma insulin levels were not altered in male (Table 1) or female (data not shown) KO mice. Fetuin KO mice demonstrate significantly lower levels of plasma FFAs and TG ($P = 0.001$) under fasting conditions than WT mice (Table 1). Fasting levels of plasma leptin are not significantly altered in fetuin KO mice compared with WT mice (Table 1). Levels of FFAs, TG, and leptin were assayed only in females due to blood sample limitations.

Increased whole-body glucose utilization and 2-DOG uptake. Euglycemic (100 mg/dl) clamps were performed on male fetuin KO mice and age-, sex-, and weight-matched WT controls to assess glucose utilization under hyperinsulinemic (100 mU · kg^{-1} · min^{-1}) conditions. Glucose infusion rates (GIRs) in fetuin KO mice were higher (113.8 ± 6.4 mg · kg^{-1} · min^{-1}) than those in WT control mice (94.4 ± 7.1 mg · kg^{-1} · min^{-1}) ($P = 0.077$) (Fig. 4*A*). No differences in plasma insulin and plasma glucose were detected between KO and WT mice (data not shown). During the last 40 min of the euglycemic clamp, a bolus of [^{14}C]2-DOG was administered to determine glucose uptake rates in individual tissues. Although no significant differences were identified between fetuin KO and control mice in the tissues sampled, an increasing trend in 2-DOG uptake was observed in gastrocnemius muscle ($P = 0.154$) and soleus and white adipose tissue ($P = 0.168$) of KO mice (Fig. 4*B*).

To assess the fate of glucose under insulin action, glycogen content was measured in heart, hindlimb, and liver at the end of the hyperinsulinemic clamp study. Hindlimb glycogen content was ~1.9-fold greater in fetuin KO mice (Fig. 4*C*), which is consistent with the increase in 2-DOG uptake. No significant differences between groups were measured in heart and liver glycogen content.

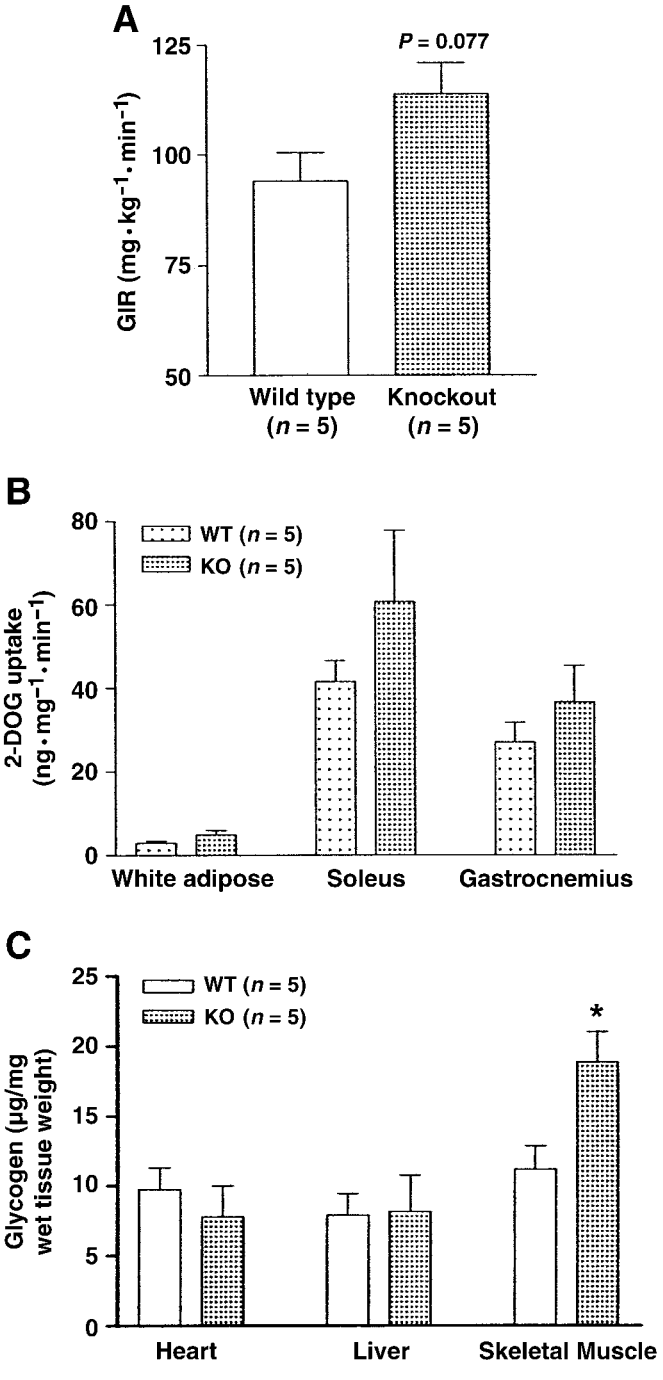

FIG. 4. Euglycemic-hyperinsulinemic clamp studies in conscious KO and WT mice. Glucose infusion rate (*A*) and 2-DOG uptake in white adipose tissue, soleus, and gastrocnemius muscles (*B*) were determined using the euglycemic-hyperinsulinemic clamp technique in fasted 12- to 16-week-old male mice. Tissue glycogen content (*C*) was assayed at the end of the euglycemic-hyperinsulinemic clamp. Results are means ± SE for five animals per genotype. *$P < 0.05$, WT vs. KO.

Obesity resistance in fetuin-null mice. HF feeding induces body weight gain and obesity (32,33) and is associated with insulin resistance (34,35). Fetuin KO and WT female mice were fed a HF diet (58.77% of calories from fat) for a period of 9 weeks. At the end of the 9-week period, WT mice on HF diet had significantly higher body weight ($P < 0.005$) than WT mice on a LF diet (Table 2). Remarkably, KO mice (at 9 weeks) remained lean, with body weights comparable to those of WT mice on a LF diet. In WT mice, the HF diet produced a 15.83% increase in body weight. Fetuin KO mice were substantially protected from diet-induced weight gain, with an average increase in body weight of only 8.44%. The total caloric intake (over 9 weeks) by WT and KO mice was not different (WTHF, 6,045 ± 180 kcal; KOHF, 5,652 ± 499 kcal). The weight-to-length ratio was significantly higher in WTHF mice ($P < 0.01$) than in KOHF mice. Total fat weight was significantly higher in WTHF mice than in

TABLE 2
Body weight parameters, blood glucose, plasma insulin in WT and KO mice fed LF or HF diets

	WTHF	WTLF	KOHF	KOLF	*P* Genotype	*P* Diet
n	11	11	11	10		
Body weight (g)	28.1 ± 0.9[a]	24.3 ± 0.9[b]	24.4 ± 1.0[b]	22.5 ± 0.9[b]	<0.01	<0.005
Weight/length (g/cm)	2.85 ± 0.09[a]	2.55 ± 0.07[b]	2.5 ± 0.1[b]	2.39 ± 0.08[b]	<0.01	<0.05
Liver weight (g)	1.07 ± 0.04	1.1 ± 0.04	1.00 ± 0.04	1.07 ± 0.03	NS	NS
Total fat weight (g)	5.8 ± 2.1[a]	3.4 ± 1.9[b]	3.7 ± 2.1[b]	2.62 ± 1.1[b]	<0.01	<0.005
Total fat (%)	20.1 ± 0.6[a]	13.5 ± 0.6[b]	14.4 ± 0.6[b]	11.4 ± 0.4[b]	<0.01	<0.005
Food intake (kcal)	6,045 ± 180[a]	3,505 ± 36[b]	5,652 ± 499[a]	3,713 ± 229[b]	NS	<0.01
Fasting glucose (mg/dl)	93.64 ± 7.1	83.64 ± 7.2	84.91 ± 3.9	80.8 ± 4.8	NS	NS
Fasting insulin (ng/ml)	0.36 ± 0.03[a] (*n*=8)	0.32 ± 0.01 (*n*=9)	0.28 ± 0.01[b] (*n*=9)	0.30 ± 0.01 (*n*=8)	<0.05	NS

Data are means ± SE. Fetuin KO and WT mice (females, 10 weeks old) were fed HF or LF diets ad libitum for 9 weeks and were monitored weekly for food intake. End-of-study body weight parameters and total caloric intake are shown. Values were dissimilar superscript letters ([a] or [b]) are significantly different from each other based on genotype or diet.

KOHF mice ($P < 0.01$). Similar results were obtained when expressed as percentage total fat (ratio of total fat weight to body weight), with KOHF mice showing significantly lower percentages ($P < 0.01$) of total fat than WTHF mice.

The effect of HF diet on insulin levels and insulin sensitivity was assessed in fetuin KO and WT mice. WTHF mice showed significantly higher fasting insulin levels ($P < 0.05$) than KOHF mice (Table 2). Fasting blood glucose concentrations did not differ in the four groups of mice. In response to an i.p. glucose load (1.5 mg/g), no impairment in glucose tolerance was observed in any of the four groups (data not shown). Although the secretory response of insulin was not altered, insulin levels at time zero and at 30 and 60 min after the GTT were significantly higher in WTHF than in KOHF mice (Fig. 5*A*). According to homeostasis model assessment (HOMA) of insulin sensitivity (fasting glucose [mmol/l] multiplied by fasting insulin [μU/ml] divided by 22.5) (36), KOHF mice demonstrated HOMA scores similar to those of WTLF mice, indicating that KOHF mice retained their insulin sensitivity (Fig. 5*B*), whereas WTHF mice showed significantly higher HOMA scores ($P < 0.05$), reflecting insulin resistance.

DISCUSSION

Decreased action of insulin in peripheral tissues is a central feature of several common pathological states, including type 2 diabetes, obesity, hypertension, and glucocorticoid excess (3,37). Improving insulin sensitivity offers a promising approach to the prevention, intervention, and treatment of these pathological conditions. Several animal models demonstrate a potential to increase insulin sensitivity, namely Pik3r1$^{-/-}$, PPAR-γ$^{+/-}$ (peroxisome proliferator–activated receptor-γ heterozygous), PTP1B Ex1$^{-/-}$ (protein tyrosine phosphatase 1B knockout), and transgenic Ucp-L mice (38–42). Fetuin has irstatin (IR inhibitory) activity and interacts with the activated IR (9,17,43). In this study, we used mice that carry two null alleles for the *Ahsg* gene to examine the hypothesis that deficiency of fetuin leads to increased IR autophosphorylation and downstream insulin signaling, thereby improving whole-body insulin sensitivity.

Fetuin KO mice exhibit increased insulin sensitivity, as evidenced by augmented phosphorylation of IR, TK activity, activation of MAPK and Akt, and enhanced glucose clearance rates. The observed increase in basal IR phosphorylation (no added insulin) and TK activity and moderate increases in insulin-stimulated IR autophosphorylation in KO mice validate the irstatin role of fetuin. The increased insulin-stimulated signaling of downstream molecules (MAPK and Akt) in KO mice also implicates increased IR activation. The discrepancy of decreased IR phosphorylation at the highest insulin concentrations (10 μmol/l) may be due to IR downregulation after in vivo insulin exposure or dose-/time-dependent effects. It may be noted that the observed dose-dependent variations are similar in WT and KO mice.

The observed changes in insulin responsiveness, ranging from mild to moderate, may be due to the altered lipid metabolism or phosphorylation status of IR and other downstream signaling molecules. Fetuin KO mice demonstrate decreased body weight and resistance to diet-induced obesity. This may be due to decreased fat stores resulting from altered lipid metabolism or increased energy expenditure. Interestingly, other mouse models of increased insulin sensitivity, such as PTP1B knockout mice, PPARγ heterozygous mice, and mice that lack the Klotho gene, also show significant reduction in size (39–41,44). On the contrary, mice that are selectively insulin resistant in muscle have an obese phenotype (45).

Oral and intraperitoneal GTTs and ITTs demonstrated increased glucose clearance and improved insulin sensitivity in fetuin KO mice; however, fasting glucose levels were not significantly altered. Improved insulin sensitivity has been shown to be associated with decreased fasting insulin levels and decreased insulin secretion in response to a glucose challenge (38,46,47). However, fetuin KO mice did not show any difference in fasting or fed insulin levels. In response to a glucose load, insulin levels were not significant different. Fetuin KO mice demonstrated improved insulin sensitivity, as assessed by ITT (0.15 units/kg). This improved sensitivity of fetuin KO mice at low insulin concentrations may be metabolically meaningful considering the fact that basal IR phosphorylation is elevated in KO mice.

Under euglycemic-hyperinsulinemic clamp conditions, whole-body glucose disposal was increased in KO mice, almost reaching statistical significance ($P = 0.077$, $n = 5$). Alhough 2-DOG uptake into muscle of KO mice only

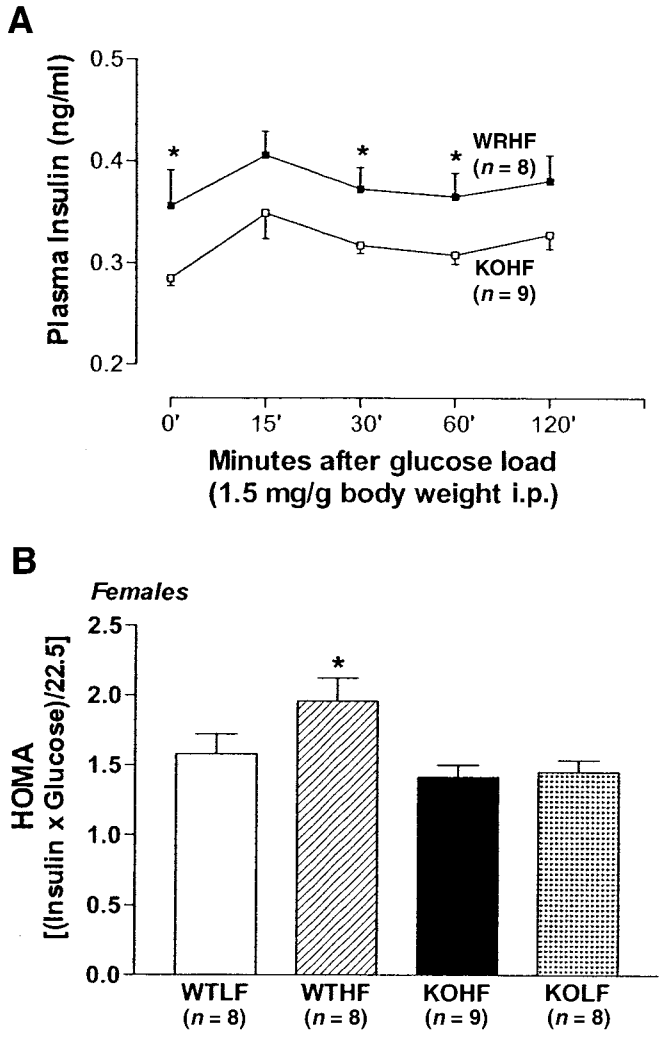

FIG. 5. Plasma insulin and HOMA in WT and KO mice fed LF or HF diets. After an overnight fast, HF- or LF-fed (9 weeks) fetuin KO and WT mice were given an i.p. glucose tolerance test (1.5 mg glucose/g), and blood glucose and plasma insulin concentrations were measured. Results are means ± SE. *A*: **P* < 0.05, WTHF vs. KOHF. *B*: **P* < 0.05, WTHF vs. WTLF, KOHF, or KOLF.

showed a trend toward increased insulin effect compared with WT mice, the glycogen content of hindlimb muscles was increased significantly in the KO mice after the hyperinsulinemic clamp study, indicating an increased shunting of infused glucose to glycogen in skeletal muscle (liver or heart glycogen content was not altered in KO mice compared with WT mice). These data support the increased glucose clearance rates observed during oral and intraperitoneal GTT. It is possible that the increased skeletal muscle glycogen content may be due to alterations in GLUT4 expression/membrane localization or modifications in glycogen metabolism.

Because an improved insulin sensitivity and lowered plasma lipid content was observed in fetuin KO mice, it was hypothesized that a high-fat diet would lead to less insulin resistance and body weight gain in KO mice compared with WT mice. KOHF mice had significantly lower body weights compared with WTHF mice, and total body fat content was significantly lower in KOHF mice. Further, KO mice maintained insulin sensitivity even after 9 weeks of HF feeding, unlike WTHF mice, which became hyperinsulinemic and less insulin sensitive, as evidenced by HOMA scores. Because KO mice lack fetuin, IR autophosphorylation can proceed more effectively, thus presumably maintaining normal glucose metabolism in spite of HF feeding. Whether the resistance to weight gain is related to the improved insulin sensitivity per se, increased basal metabolic rate (BMR), or other non–BMR-related energy expenditures is yet to be understood.

A second member of the fetuin family, fetuin-B, was identified recently (48). Whether fetuin-B shares irstatin activity with fetuin-A and whether such fetuin redundancy could protect against the deleterious effects of gene deletion is not known. Interestingly, mice deficient in PTP1B demonstrate a phenotype similar to that of fetuin KO mice, namely increased insulin sensitivity and IR phosphorylation, decreased adiposity, and resistance to weight gain (40,41). This was not unexpected, since both fetuin and PTP1B decrease IR phosphorylation.

In summary, this study provides the first direct evidence that fetuin plays a critical role in clearance/uptake of glucose from blood and in modulating insulin sensitivity. Although the exact mechanism for fetuin's role in regulating insulin sensitivity is not understood, it may be mediated by modulation of phosphorylation status of IR and subsequent downstream signaling and by the alteration of lipid metabolism. Furthermore, these studies identify fetuin as a novel therapeutic target in the treatment of type 2 diabetes, obesity, and other insulin-resistant conditions.

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