

Regulation of Resistin Expression and Circulating Levels in Obesity, Diabetes, and Fasting

Michael W. Rajala,¹ Yong Qi,² Hiral R. Patel,² Nobuhiko Takahashi,² Ronadip Banerjee,² Utpal B. Pajvani,¹ Madhur K. Sinha,³ Ronald L. Gingerich,³ Philipp E. Scherer,¹ and Rexford S. Ahima²

Resistin was originally reported as an adipose tissue-specific hormone that provided a link between obesity and diabetes. Resistin protein level was elevated in obese mice and decreased by insulin-sensitizing thiazolidinediones. Immunoneutralization of resistin improved insulin sensitivity in diet-induced obese mice, while the administration of exogenous resistin induced insulin resistance. More recently, we have shown that ablation of the resistin gene in mice decreased fasting glucose through impairment of gluconeogenesis, while resistin treatment in these knockout mice increased hepatic glucose production. However, the link between resistin and glucose homeostasis has been questioned by studies demonstrating reduced, rather than increased, resistin mRNA expression in obese and diabetic mice. To better understand the regulation of resistin, we developed a sensitive and specific RIA resistin that could accurately measure serum resistin levels in several mouse models. We show that while resistin mRNA is indeed suppressed in obese mice, the circulating resistin level is significantly elevated and positively correlated with insulin, glucose, and lipids. Both resistin mRNA expression and protein levels in *Lep^{ob/ob}* mice are suppressed by leptin treatment in parallel with reductions in glucose and insulin. In wild-type mice, serum resistin increases after nocturnal feeding, concordant with rising levels of insulin. Resistin mRNA and protein levels decline in parallel with glucose and insulin during fasting and are restored after refeeding. We performed clamp studies to determine whether resistin is causally related to insulin and glucose. Adipose resistin expression and serum resistin increased in response to hyperinsulinemia and further in response to hyperglycemia. Taken together, these findings suggest that the nutritional regulation of resistin and changes in resistin gene expression and circulating levels in obesity are mediated, at least in part, through insulin and glucose. *Diabetes* 53:1671–1679, 2004

From the ¹Department of Cell Biology and Diabetes Research and Training Center, Albert Einstein College of Medicine, Bronx, New York; the ²Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, University of Pennsylvania, Philadelphia, Pennsylvania; and ³Linco Research, St. Charles, Missouri.

Address correspondence and reprint requests to Rexford S. Ahima, MD, PhD, University of Pennsylvania School of Medicine, Division of Endocrinology, Diabetes and Metabolism, 764 Clinical Research Building, 415 Curie Blvd., Philadelphia, PA 19104. E-mail: ahima@mail.med.upenn.edu.

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DEXA, dual-energy X-ray absorptiometry; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; TBS, Tris-buffered saline; TZD, thiazolidinedione.

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Despite many recent studies, the link between obesity and the development of insulin resistance and diabetes remains unclear (1). It is now recognized that in addition to storing energy in the form of triglyceride, adipose tissue secretes a variety of proteins and other molecules actively involved in energy, glucose, and lipid homeostasis (2). Recent studies appear to indicate that the insulin-sensitizing effects of peroxisome proliferator-activated receptor- γ agonists such as thiazolidinediones (TZDs), a class of drugs widely used clinically for the treatment of type 2 diabetes, may require adipose tissue and may in fact be derived from modulating the levels of a number of adipocyte-derived secretory proteins (3). These so-called “adipokines” have been shown to affect insulin sensitivity in a number of different tissues (4). Resistin, also known as FIZZ3 (found in inflammatory zone 3) and ADSF (adipocyte-secreted factor), was initially identified in a screen for adipocyte-specific transcripts downregulated by treatment with TZDs (5,6). It belongs to a novel family of cysteine-rich proteins, each with a unique tissue distribution of expression (5–7). In rodents, resistin is predominantly expressed in white adipose tissue (5–7). It was first suggested that resistin might provide a link between obesity and diabetes (5). The administration of recombinant resistin resulted in impaired glucose tolerance in wild-type mice, while immunoneutralization of endogenous resistin improved insulin sensitivity in diet-induced obese (DIO) mice (5). Additionally, resistin mRNA levels were suppressed in 3T3-L1 adipocytes and mouse adipose tissue by TZDs, potentially providing one of the mechanisms by which TZDs act to improve insulin sensitivity (5). Serum resistin was higher in *Lep^{ob/ob}* and DIO mice (5). Furthermore, the acute administration of resistin in euglycemic-hyperinsulinemic pancreatic clamp studies resulted in decreased insulin sensitivity primarily at the level of the liver, by decreasing the insulin-mediated suppression of hepatic gluconeogenesis (8). We have recently confirmed the latter by showing that ablation of the resistin gene in mice lowered fasting glucose (9). Conversely, resistin treatment in these knockout mice increased glucose level, primarily by enhancing hepatic glucose production (9).

Despite the above evidence, the link between resistin in glucose homeostasis has been questioned as a result of studies showing a reduction in resistin mRNA levels in murine obesity models (10,11), as well as increased resis-

tin mRNA expression in response to TZD treatment (10). Moreover, resistin mRNA expression was lower in rats rendered obese on a fructose-rich diet as compared with regular diet-fed animals (12). Since resistin is secreted by adipose tissue, present in the circulation, and presumed to act at distant tissues (13), the ability to accurately measure serum resistin is crucial to the elucidation of its biological function. We hypothesized that resistin gene expression may not correlate with circulating levels. Thus, the primary purpose of this study was to produce a specific and accurate radioimmunoassay (RIA) to enable a comparison of resistin protein levels and mRNA expression in normal and obese mice. Furthermore, we tested the hypothesis that the changes in resistin mRNA and protein levels in normal and obese/diabetic animals were mediated through insulin and/or glucose.

RESEARCH DESIGN AND METHODS

C57Bl/6J and *Lep^{ob/ob}* mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and fed regular or high-fat diets as described below. Mice deficient in resistin were generated by replacing the coding exons of the resistin gene with the reporter gene *LacZ* containing a nuclear localization signal (9). Resistin mRNA and protein expression in white adipose tissue (WAT) were completely ablated (9). *ap2/D^{Tx}* mice, whose adipose tissue is virtually eliminated, were kindly provided by Reed Graves (14). The mice were housed $n = 5$ /cage on a 12-h light-dark cycle (lights on at 0600) and allowed free access to a high-fat (45%) diet (# D12451; Research Diets, New Brunswick, NJ) and water. All experiments were performed in accordance with regulations and recommendations of the institutional animal use committees.

Resistin reagents. Expression, purification, and characterization of recombinant resistin were performed as previously described (8). Anti-resistin antibody was generated as described in reference 11. Affinity purification of resistin antibody was performed as follows: recombinant mouse resistin (500 μ g) in Hepes (pH 8.5) and 500 mmol/l NaCl incubated with 250 μ l activated CNBr Sepharose (Amersham-Pharmacia) for 1 h. Subsequently, 100 μ l of 1 mmol/l Tris (pH 8.0) was added and incubated at room temperature for 30 min to saturate excess binding sites. The resin was washed two times with Tris-buffered saline (TBS), mock eluted with 200 mmol/l glycine (pH 2.5), and re-equilibrated with TBS. Anti-resistin rabbit polyclonal serum (1 ml) was added and bound overnight at 4°C. The serum was eluted, and the column was washed with 10 column volumes of TBS. Affinity purified antibody was eluted with one column volume of 200 mmol/l glycine (pH 2.5) directly into 1 mmol/l Tris (pH 8.0). Due to the absence of any endogenous tyrosine residues for iodination, the last residue in the resistin cDNA was mutated to tyrosine by PCR and subcloned into the expression construct pFM1 (8). The new construct (pFM1-A12 α Δ Y) was transfected into 293T HEK cells and sorted for high-level expression, and the tyrosine mutant was biochemically purified from serum-free media as previously described (8).

Resistin RIA. Recombinant mouse resistin tyrosine mutant was radiolabeled with ¹²⁵I by the chloramine-T method and purified by high-performance liquid chromatography. The lowest measurable concentration of resistin was determined by repeated within-run analysis of various resistin concentrations. The specificity of the assay was demonstrated using sera from 24-week wild-type, 24-week resistin-null, and 23-week *ap2/D^{Tx}* and wild-type mice. The only detectable cross-reactivity was with mouse RELM (resistin-like molecule)- β (0.2%) and mouse leptin (0.5%). The assay sensitivity was 3 ng/ml with inter- and intra-assay coefficients of variation <6% at 48 and 156 ng/ml concentrations. The recovery of exogenously added mouse resistin (1–25 ng/ml) in mouse serum ranged between 83 and 127%. Dilution and linearity experiments demonstrated 75–133% variation between observed and expected levels. Measurement of serum resistin were performed using the RIA based on bacterially produced resistin standards (Linco Research #8096) and multiplied by a correction factor of 4.3 to account for the differential sensitivity obtained with standards expressed in mammalian cells (8,15). Blood was collected via cardiac puncture, and serum was separated and stored at –80°C. Resistin was measured in duplicate 50- or 100- μ l samples using the mouse resistin RIA (Linco Research).

Measurement of other hormones and metabolites. Insulin was measured in duplicate 5- μ l samples by enzyme-linked immunosorbent assay (ELISA) (CrystalChem, Chicago, IL) (16). The limit of detection and coefficient of variation of insulin were 0.1 ng/ml and 5.8%, respectively. Serum leptin was measured by ELISA, and corticosterone was measured using a rodent RIA

(ICN Chemicals, Temecula, CA) as previously described (16,17). Triglyceride was measured using an enzymatic colorimetric assay (Stanbio Laboratories, Boerne, TX).

Changes in serum resistin during ad libitum feeding. Male and female C57Bl/6J mice, aged 12 weeks, were housed as described and fed a regular diet ad libitum. Groups of mice ($n = 6–8$ /sex) were killed at 0200, 0800, 1400, 1800, and 2200, blood was obtained via cardiac puncture, and serum was stored at –20°C. Glucose was measured in tail blood using a glucometer (One Touch Ultra II; Lifescan, Milpitas, CA). Resistin was measured in duplicate 50- μ l samples by RIA, and insulin was measured as described.

Effects of fasting and refeeding on resistin mRNA and protein. Male C57Bl/6J mice were fed a regular diet ad libitum or deprived of food for 12, 24, 36, or 48 h, starting from 0800. An additional cohort of mice was deprived of food for 48 h and then refed for 48 h. The mice were anesthetized, and blood was collected via cardiac puncture. Inguinal (subcutaneous), epididymal, and perirenal WAT depots were dissected, frozen in liquid nitrogen, and stored at –80°C. For processing, the samples were immersed in cold extraction buffer (20 mmol/l Tris-HCl, pH 7.5, 5 mmol/l EDTA, 10 mmol/l KCl, and 1 mmol/l phenylmethylsulfonyl fluoride) and sonicated for 20 s. The resulting homogenate was centrifuged at 4°C at 14,000g to separate lipids, lysate, and insoluble material. The lipid was removed, and Triton-X-100 was added to 1% final concentration to the lysate. Samples were spun again at 14,000g, and the supernatant was used for resistin measurements. Serum resistin, insulin, leptin, corticosterone, and triglycerides were measured. RNA was extracted from WAT depots, and resistin and β -actin were measured by Northern blot and quantified by Phosphorimager (5,6).

Resistin mRNA and protein levels in diet-induced and genetic obesity. Male C57Bl/6J mice were housed $n = 5$ /cage and fed regular (LabDiet, Richmond, IN), low-fat, or high-fat diets (Research Diets), starting from age 4 weeks until 24 weeks ($n = 10–14$ per diet) (16,18). The regular diet contained 4% fat, 23.4% protein, and 49.9% carbohydrate (4 kcal/g); the low-fat diet contained 10% fat, 20% protein, and 70% carbohydrate (3.8 kcal/g); and the high-fat diet contained 45% fat, 20% protein, and 35% carbohydrate (4.7% kcal/g). Body composition was assessed at 24 weeks by dual-energy X-ray absorptiometry (DEXA) (PIXImus DEXA; General Electric, Madison, WI) (19). Glucose was measured in tail blood using a glucometer (One Touch Ultra II; Lifescan), and the mice were killed between 0800 and 1100. Blood was collected via cardiac puncture. Resistin, insulin, and leptin were measured by RIA. Epididymal WAT was harvested, total RNA was extracted, and resistin and β -actin mRNA levels were measured by Northern blot. Female *Lep^{ob/ob}* and wild-type mice (ages 6, 12, and 20 weeks, respectively, $n = 7–9$) were fed a regular diet. Glucose was measured in tail blood using a glucometer (One Touch Ultra II; Lifescan), and body fat was measured by DEXA, after which the mice were killed and serum was collected for resistin, insulin, leptin measurement. Resistin and β -actin mRNA levels were measured in perigonadal WAT.

Effect of leptin treatment on resistin mRNA and protein levels in *Lep^{ob/ob}* mice. We tested the hypothesis that an improvement in insulin sensitivity by leptin treatment in *Lep^{ob/ob}* mice would decrease resistin mRNA and protein levels. Female *Lep^{ob/ob}* mice received a daily intraperitoneal injection of murine leptin (2 mg/kg) (A.F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases) or PBS for 2 weeks. A third group was pair-fed (food restricted) to leptin treatment ($n = 6–8$). Body weight and food intake were measured daily. Rectal temperature, tail glucose, and body fat (by DEXA) were measured before and at the end of the experiment. The mice were killed, cardiac blood drawn, and perigonadal fat excised and frozen for mRNA studies. Resistin, insulin, triglycerides, and corticosterone were measured.

Glucose clamp in wild-type mice. We investigated the causal relationship between insulin and glucose and between resistin gene expression and protein levels in clamp studies. Male C57Bl/6J mice were anesthetized by intraperitoneal injection with sodium pentobarbital. Two catheters (Micro-Renathane 0.025-inch outer diameter; Braintree Scientific, Braintree, MA) were inserted into the jugular vein and secured with 4-0 silk sutures. The catheters are tunneled subcutaneously, exteriorized dorsally, and filled with sterile saline. Clamp studies were conducted 5 days postoperatively. The mice were fasted for 6 h (from 0800) and restrained via the tail as described (3). One group of mice ($n = 5$) was subjected to a hyperinsulinemic-euglycemic clamp. After basal sampling, a bolus injection of 40 mU/kg human regular insulin (Humulin; Eli Lilly, Indianapolis, IN) was administered intravenously, followed by a continuous insulin infusion at a rate of 20 mU \cdot kg⁻¹ \cdot min⁻¹. Tail blood samples (2 μ l) were collected at 10-min intervals for glucose measurement, and 30% glucose was infused at variable rates to maintain blood levels at 120–140 mg/dl for 120 min. Additional 10- μ l samples were collected at 30-min intervals for insulin measurement. A second group ($n = 5$) was subjected to a hyperinsulinemic hyperglycemic clamp (300–350 mg/dl), and the third group

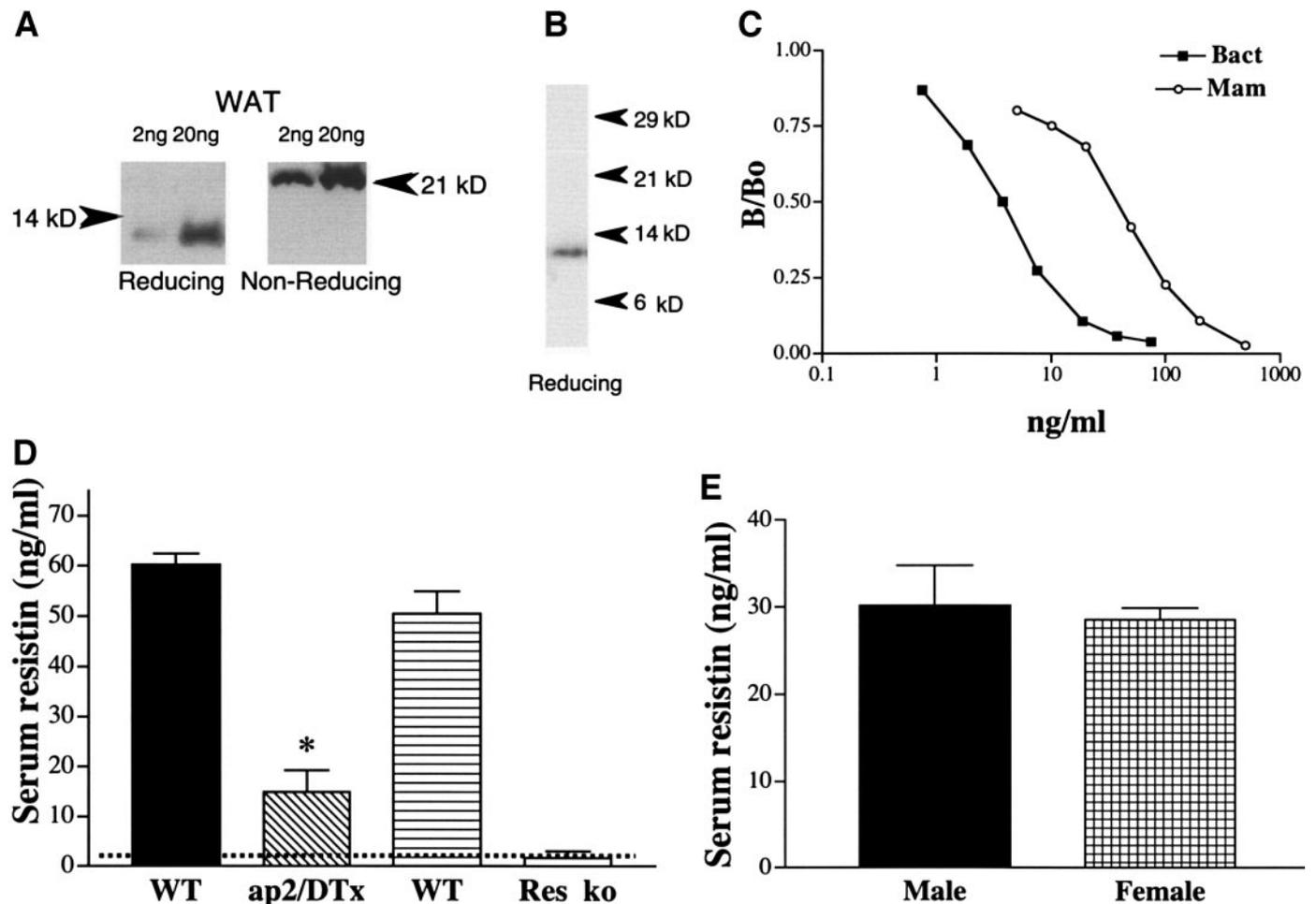


FIG. 1. *A:* Immunoblotting of resistin in epididymal fat from a wild-type mouse under reducing and nonreducing conditions. The amount of total protein in the lysate is shown. Molecular weight markers are noted by arrows. *B:* Immunoblotting of resistin in wild-type size-fractionated mouse serum. *C:* Binding curves of resistin protein expressed in bacteria (Bact) and HEK 293T (Mam) cells using affinity-purified antibodies. *D:* RIA measurement of resistin in sera from resistin-null (Res ko) and lipodystrophic ap2/DTx mice fed a high-fat (45%) diet. WT for Resko (▨), ap2/DTx (▩). Data are means \pm SE, $n = 4$. *E:* RIA measurements of resistin at 0800–0900 in male and female wild-type C57Bl/6J mice fed a regular diet, $n = 10$.

($n = 5$) received PBS infusion. At the end of the clamp, animals were killed and epididymal fat was excised, frozen immediately, and stored at -80°C for later analysis.

Statistics. Data are presented as means \pm SE. The changes in resistin and various parameters were analyzed by ANOVA, and pairwise differences were assessed using Newman-Keuls test (GraphPad Prism, San Diego, CA). Serum resistin levels in DIO and *Lep^{ob/ob}* mice were compared with body fat, insulin, and glucose levels by Pearson's correlation (GraphPad Prism). $P < 0.05$ was considered significant.

RESULTS

Resistin RIA. To demonstrate the specificity of the anti-resistin antibody, we show the detection of resistin in epididymal fat from wild-type mice that migrates as an apparent dimer under nonreducing conditions and a monomer under reducing conditions on SDS-PAGE (Fig. 1A). Similarly, immunoblotting of serum under reducing conditions reveals resistin migrating as a specific, ~ 10 -kDa band (Fig. 1B). The standard curve of the RIA based on resistin protein standards produced in bacteria was parallel but shifted to the left of the assay based on mammalian-produced resistin (Fig. 1C). The reason for this difference in relative potencies between the bacterial and mammalian protein standards is unclear. The standard curves looked identical whether the antibody was affinity

purified or not. Affinity purified antibody was used for data in Fig. 1C. The specificity of the assay is confirmed in Fig. 1D, which shows resistin to be undetectable in serum samples from resistin-null mice and markedly decreased in lipodystrophic ap2/DTx mice. Figure 1E shows that there were no significant differences between male and female mice in these studies.

Resistin levels in ad libitum-fed and fasted mice. Figure 2A shows the changes in serum resistin levels during ad libitum feeding. Resistin tended to increase after the onset of nocturnal feeding, with a peak level at 0200, followed by a decline during the day. Serum resistin at 0200 was significantly higher than at 1800 ($P < 0.05$). Interestingly, the changes in serum resistin levels paralleled the nocturnal increase in insulin and decline during the day (Fig. 2B). Glucose peaked at 0200 (178 ± 8.5 mg/dl) and declined to a nadir (103 ± 6.3 mg/dl; $P < 0.01$) at 1800. The temporal patterns of resistin, insulin, and glucose were similar in age-matched female C57Bl/6J mice (data not shown).

We examined the effects of fasting on resistin expression in epididymal, subcutaneous, and perirenal WAT and on serum levels. Resistin mRNA and protein levels were

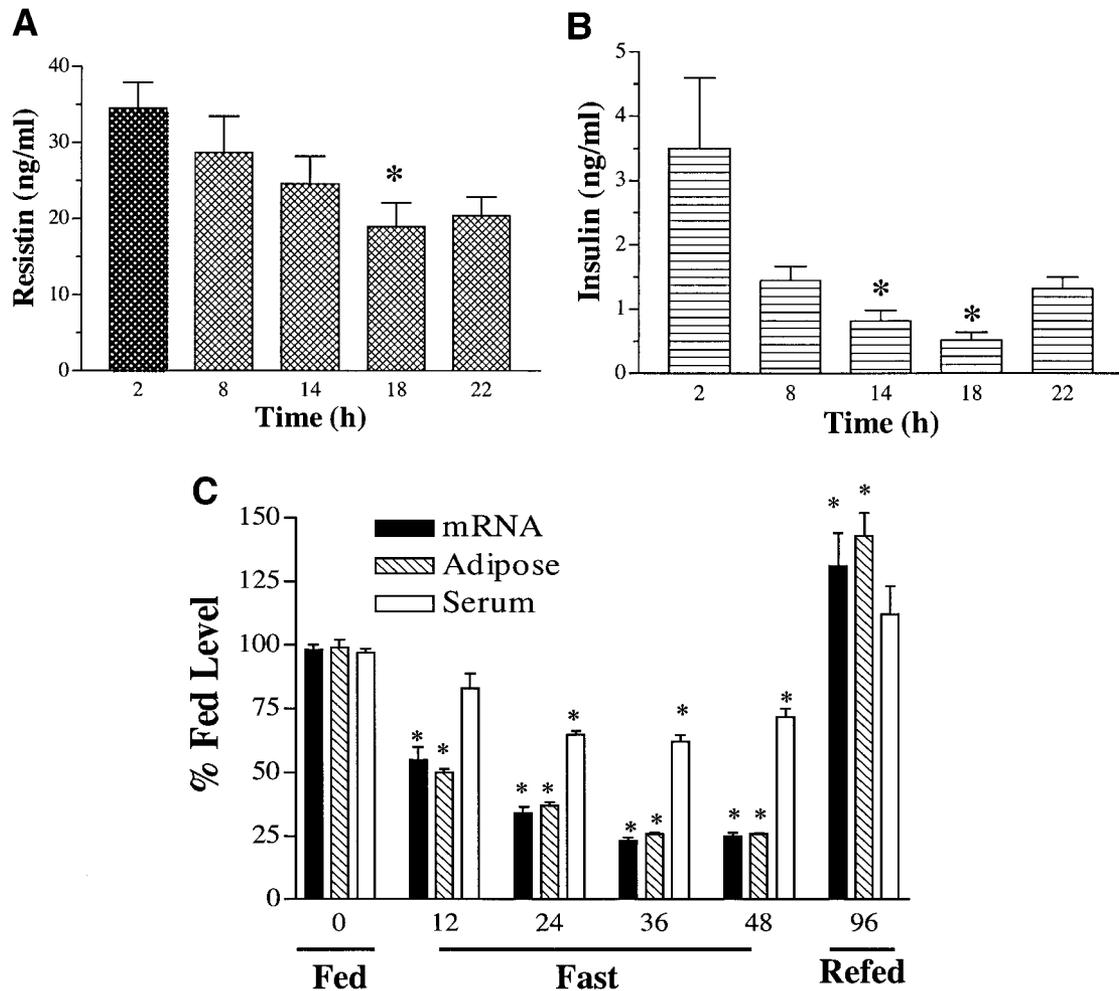


FIG. 2. Changes in serum levels of resistin (A) and insulin (B) in ad libitum-fed C57BL/6J mice kept in 12-h light-dark cycles (lights on 0600). Groups of mice were killed at 0200, 0800, 1400, 1800, and 2200. Data are means \pm SE, $n = 6-8$. C: Nutritional regulation of resistin mRNA in epididymal WAT and protein levels in epididymal WAT and serum in wild-type C57BL/6J mice. Data are means \pm SE, $n = 5$. $P < 0.05$ vs. fed level.

decreased in epididymal WAT within 12 h of food deprivation (Fig. 2C). After 48 h of fasting, resistin mRNA and protein levels had dropped to $\sim 25\%$ of fed levels (Fig. 2C). Expression levels rebounded in response to refeeding (Fig. 2C). Similar changes in resistin mRNA and protein levels were observed in perirenal and inguinal (subcutaneous) WAT depots (data not shown). The serum concentration of resistin declined significantly after the 36- to 48-h fast. This decline was restored within 48 h of refeeding (Fig. 2C). Food deprivation resulted in weight loss, reduced levels of glucose, triglyceride, insulin, and leptin as

TABLE 1
Effects of fasting and refeeding on blood chemistry

	Fed	Fasted	Refed
Body weight (g)	25.7 \pm 1.2	20.6 \pm 0.9*	26.1 \pm 1.4
Glucose (mg/dl)	127 \pm 9.8	65.2 \pm 7.5*	133 \pm 12.1
Triglycerides (mg/dl)	87 \pm 5.6	65.3 \pm 4.1*	95.7 \pm 3.8
Resistin (ng/ml)	29.2 \pm 1.4	15.2 \pm 5.2*	47.5 \pm 6.6*
Leptin (ng/ml)	3.92 \pm 0.21	1.37 \pm 0.03*	6.09 \pm 1.4*
Insulin (ng/ml)	1.09 \pm 0.12	0.28 \pm 0.08*	2.15 \pm 0.45*
Corticosterone (ng/ml)	84.7 \pm 7.8	244 \pm 36.2*	97.3 \pm 11.2

Data are means \pm SE, $n = 8$. Male C57BL/6J mice were fed a standard diet ad libitum or deprived of food for 48 h. Another group was deprived of food for 48 h and refed for 48 h. * $P < 0.05$ vs. fed.

well as increased levels of corticosterone (Table 1). Refeeding restored body weight, glucose, corticosterone, and triglycerides, while insulin and leptin rebounded above fed levels (Table 1).

Resistin mRNA expression and serum concentrations in *Lep^{ob/ob}* and DIO mice. We investigated the changes in resistin mRNA and protein levels in obese mice. Resistin mRNA expression in WAT from DIO mice was suppressed (Fig. 3A), while serum levels of resistin were markedly elevated (Figs. 3B). In agreement with previous reports (16,18), body fat increased significantly in high-fat-fed (37.6 ± 1.5 g; $P < 0.001$) and low-fat-fed (30.8 ± 0.95 g; $P < 0.05$) mice, compared with regular diet-fed mice (27.3 ± 0.38 g). Glucose, insulin, and leptin were significantly elevated in high-fat-fed mice (Table 2).

Resistin mRNA expression in WAT from *Lep^{ob/ob}* mice was significantly lower than that from wild-type mice (Fig. 3C), yet serum resistin levels were significantly higher in obese diabetic mice (Fig. 3D). The percentage elevation of serum resistin in the *Lep^{ob/ob}* mice over the control values tended to increase further as the mice aged from 6 to 20 weeks, although this trend was not significant ($r = 0.307$; $P = 0.126$). *Lep^{ob/ob}* mice were heavier than wild-type mice at 6 weeks (30.2 ± 1.3 vs. 18.4 ± 0.9 g; $P < 0.0001$) and became massively obese by 20 weeks (51.3 ± 3.8 vs. $28.7 \pm$

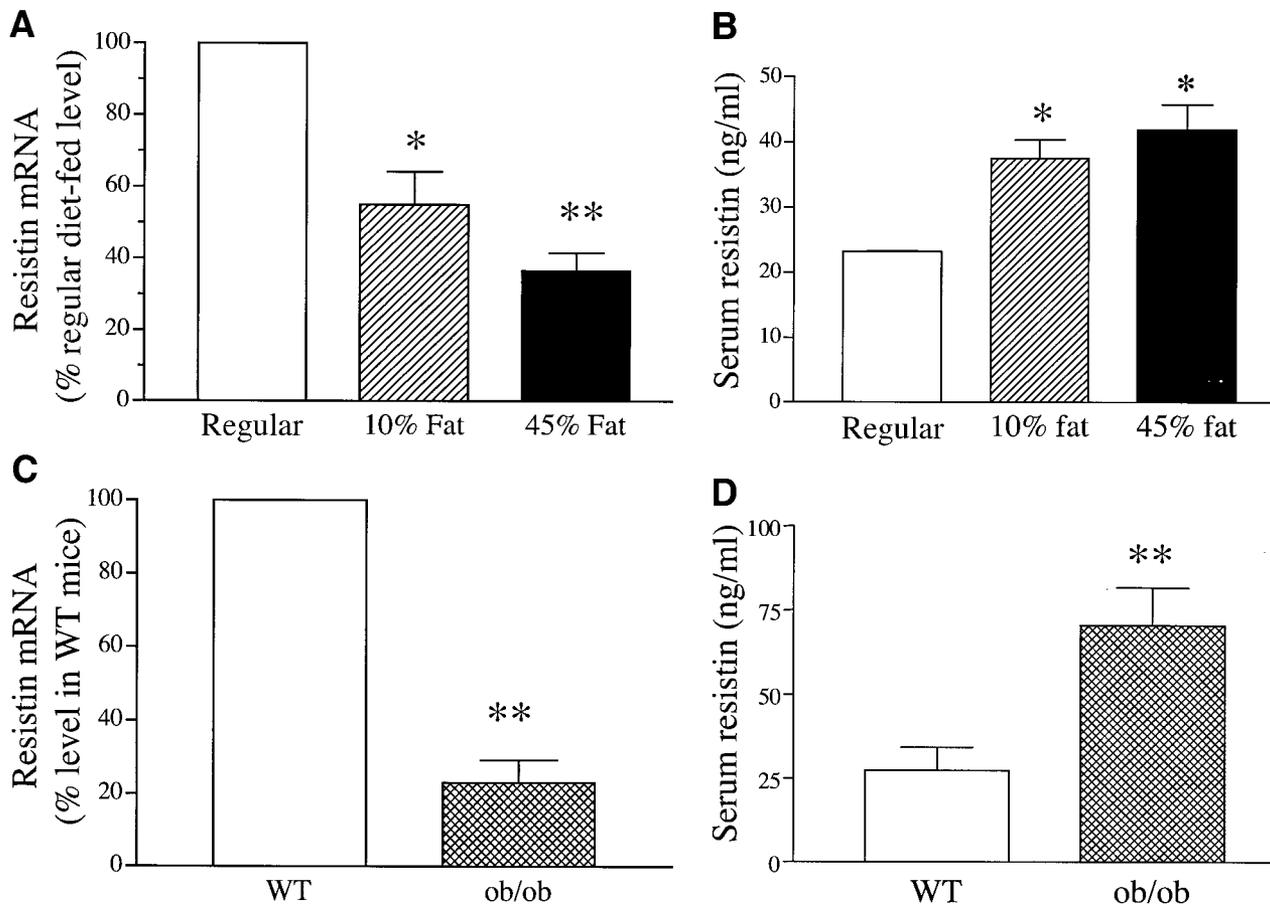


FIG. 3. Resistin mRNA expression in epididymal WAT (A) and serum resistin (B) levels in wild-type mice fed a high-fat (45%), low-fat (10%), or regular diet. Data are means \pm SE, $n = 10$ –14. * $P < 0.01$, ** $P < 0.001$ vs. regular diet. Resistin mRNA levels in epididymal WAT (C) and serum resistin (D) levels in 12-week *Lep^{ob/ob}* and wild-type mice. Data are means \pm SE, $n = 22$ in wild-type and $n = 26$ in *Lep^{ob/ob}* mice. * $P < 0.0001$ vs. wild-type.

1.6 g; $P = 0.0002$). Aging (6–20 weeks) in *Lep^{ob/ob}* mice was strongly correlated with body fat ($r = 0.78$; $P < 0.0001$), insulin ($r = 0.748$; $P < 0.0001$), and glucose ($r = 0.718$; $P < 0.0001$). Similarly, body fat ($r = 0.572$; $P = 0.01$) and insulin ($r = 0.53$; $P = 0.011$) increased significantly in aged wild-type mice.

Correlation of serum resistin with body fat, glucose, and insulin in DIO and *Lep^{ob/ob}* mice. Serum resistin level was not significantly correlated to body fat in wild-type mice fed regular ($r = 0.25$; $P = 0.48$) or low-fat ($r = 0.049$; $P = 0.87$) diets. In contrast, resistin was strongly correlated to body fat in mice fed a high-fat diet ($r = 0.710$;

$P = 0.009$) as well as when the relationship between serum resistin and body fat was examined across lean and DIO mice ($r = 0.72$; $P < 0.0001$) (Fig. 4A). Similarly, resistin was positively correlated to insulin ($r = 0.35$; $P = 0.037$) (Fig. 4B), glucose ($r = 0.55$; $P = 0.0005$) (Fig. 4C), leptin ($r = 0.77$; $P < 0.0001$), and triglyceride ($r = 0.42$; $P = 0.01$) levels when evaluated across lean and DIO mice. We also examined the associations between serum resistin and body fat (Fig. 4D), glucose (Fig. 4E), and insulin (Fig. 4F) from *Lep^{ob/ob}* ($n = 26$) and wild-type ($n = 22$) mice. There were strong positive correlations between resistin and body fat ($r = 0.65$; $P = 0.0004$), insulin ($r = 0.62$; $P = 0.0007$), and glucose ($r = 0.49$; $P = 0.01$) in *Lep^{ob/ob}* mice. However, as was the case in the lean controls of DIO mice, there was no significant correlation between resistin and body fat ($r = 0.126$; $P = 0.57$), insulin ($r = 0.214$, $P = 0.33$), or glucose ($r = 0.040$; $P = 0.89$) in the wild-type mice (Figs. 4D–F).

Resistin mRNA and protein levels are reduced by leptin. *Lep^{ob/ob}* mice are well known to have elevated glucose and insulin levels (20,21). Leptin improves insulin sensitivity in this model (20,21); hence, we administered leptin to *Lep^{ob/ob}* mice to evaluate the interrelationships among insulin, glucose, resistin gene expression, and protein levels. Leptin treatment significantly decreased food intake, body weight, and percentage of fat and increased body temperature, in agreement with previous

TABLE 2
Effects of various diets on metabolic parameters in C57BL/6J mice

	Standard	Low fat	High fat
Body weight (g)	27.8 \pm 0.38	31.6 \pm 1.05	38.5 \pm 1.3*
Body fat (%)	15.6 \pm 1.39	27.03 \pm 1.85*	34.13 \pm 0.6*†
Resistin (ng/ml)	23.2 \pm 0.16	37.6 \pm 2.83*	42.02 \pm 2.8*
Leptin (ng/ml)	5.74 \pm 0.24	16.1 \pm 2.2*	20.4 \pm 0.8*
Insulin (ng/ml)	1.82 \pm 0.4	3.49 \pm 0.76*	4.8 \pm 0.97*
Glucose (mg/dl)	132 \pm 7.3	195 \pm 12.4*	202 \pm 6.8*
Triglycerides (mg/dl)	81.2 \pm 2.6	96.4 \pm 5.1*	99.4 \pm 3.4*

Data are means \pm SE, $n = 12$. Male C57BL/6J mice were fed a standard, low-fat (10%), or high-fat (45%) diet from 4 to 24 weeks. * $P < 0.05$ vs. standard diet; † $P < 0.05$ vs. low-fat diet.

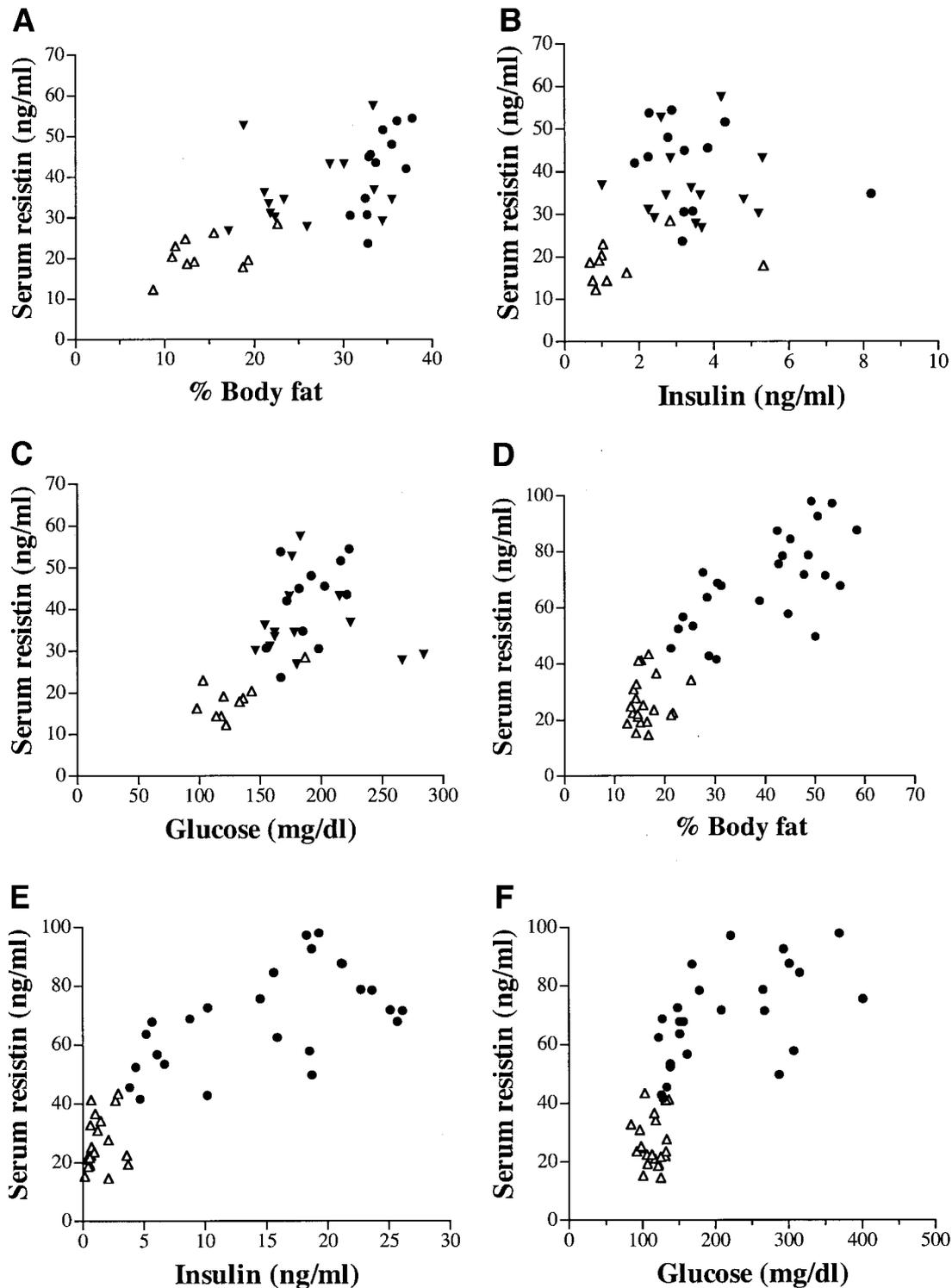


FIG. 4. Relationships between serum resistin concentration and various parameters in C57Bl/6J and *Lep^{ob/ob}* mice. Resistin was positively correlated with percentage body fat ($r = 0.72$; $P < 0.0001$) (A), insulin ($r = 0.35$; $P = 0.037$) (B), and glucose ($r = 0.55$; $P = 0.0005$) (C), when examined across lean mice fed regular (Δ) and low-fat (10%) (\blacktriangle) diets and DIO mice fed high-fat (45%) diet (\bullet), $n = 10$ –14. Resistin was also positively and strongly correlated with percentage body fat ($r = 0.65$; $P = 0.0004$) (D), insulin ($r = 0.62$; $P = 0.0007$) (E), and glucose ($r = 0.49$; $P = 0.01$) (F) in *Lep^{ob/ob}* mice (\bullet), but not in wild-type mice (Δ).

reports (20–22) (Table 3). Moreover, leptin decreased insulin, glucose, and triglyceride levels (Table 3). The ratio of insulin to glucose was 0.08 in PBS treatment and was reduced to 0.05 by leptin, consistent with improved insulin response. Corticosterone was decreased by leptin, although the change was not significant (Table 3). Resistin

mRNA expression (Fig. 5A), serum level (Fig. 5B), and WAT resistin protein (data not shown) decreased in parallel with the fall in glucose and insulin in leptin-treated animals. Pair-feeding decreased body weight and fat but had little effect on glucose, insulin, and triglyceride levels (Fig. 5A and B; Table 3). Moreover, in contrast to leptin,

TABLE 3
Effect of leptin treatment in *ob/ob* mice

	PBS	Leptin	Pair fed
Body weight (g)	54.8 ± 1.1	49.3 ± 0.5*	50.1 ± 0.65*
Body fat (%)	46.7 ± 1.3	37.1 ± 0.9*	39.2 ± 0.5*
Food intake/day	6.7 ± 0.8	3.14 ± 0.52*	3.16 ± 0.4*
Temperature (°C)	35.1 ± 0.1	36.9 ± 0.07*	34.8 ± 0.17†
Glucose (mg/dl)	247 ± 10.3	162 ± 5.2*	195 ± 9.8
Insulin (ng/ml)	20.5 ± 2.4	8.76 ± 1.3*	14.7 ± 1.1
Triglyceride (mg/dl)	136 ± 4.5	94 ± 1.3*	127 ± 4.6
Corticosterone (ng/ml)	254 ± 15	188 ± 11.3	203 ± 5.5

Data are means ± SE, $n = 6-8$. *Lep^{ob/ob}* mice were treated with leptin or PBS for 14 days. Pair-fed mice were restricted to the daily intake of leptin-treated mice. * $P < 0.05$ vs. PBS; † $P < 0.05$ vs. leptin.

pair-feeding had no significant effect on resistin mRNA expression and protein levels (Fig. 5A and B).

Resistin mRNA and protein are increased by insulin and glucose under clamp conditions. We examined the causal relationship among resistin, insulin, and glucose in wild-type mice using the clamp technique (Table 4). Resistin mRNA level was increased by 20% in response to hyperinsulinemia and was further increased by 25% in response to hyperglycemia (Fig. 5C). Similarly, both insulin and glucose increased serum resistin level (Fig. 5D). There was a slight but insignificant increase in WAT resistin protein content in response to insulin and glucose (data not shown).

DISCUSSION

Resistin was originally named for its putative role as a mediator of insulin resistance in rodents (5). Circulating

TABLE 4
Parameters for glucose clamp studies in C57B/6J mice

	PBS	Insulin euglycemia	Insulin hyperglycemia
Body weight (g)	27 ± 1.0	26.3 ± 0.8	27.8 ± 1.1
Basal glucose (mg/dl)	132 ± 11.1	129 ± 8.3	134 ± 11.1
Basal insulin (ng/ml)	1.1 ± 0.2	0.98 ± 0.14	0.96 ± 0.31
Clamp glucose (mg/dl)	131 ± 4.8	129 ± 2.1	336 ± 4.0*†
Clamp insulin (ng/ml)	0.9 ± 0.1	299 ± 46.7	315 ± 36
Glucose infusion (mg · kg ⁻¹ · min ⁻¹)	—	96.4 ± 4.8	137 ± 9.6†

Data are means ± SE, $n = 5$. Male C57BL/6J mice (12 weeks) were subjected to hyperinsulinemic-euglycemic or hyperglycemic clamps. Control mice received PBS infusion. * $P < 0.05$ vs. PBS; † $P < 0.05$ vs. euglycemia.

resistin level measured by immunoblotting was elevated in obese mice (5). Importantly, administration of recombinant resistin impaired glucose tolerance, whereas immunoneutralization of resistin in DIO mice lowered glucose levels (5). This effect of resistin was subsequently corroborated by a study in which infusion of resistin or the related protein, RELM- β , acutely stimulated hepatic glucose output in rats under pancreatic clamp conditions (8). Moreover, transgenic overexpression of resistin impaired glucose transport in skeletal muscle, while treatment with recombinant resistin decreased insulin-mediated glucose transport in myotubes (23,24). Recently, we have shown that ablation of the resistin gene in mice lowered fasting glucose by blunting gluconeogenesis (9). In contrast, treatment of the knockout mice with recombinant resistin increased glucose by enhancing hepatic production (9).

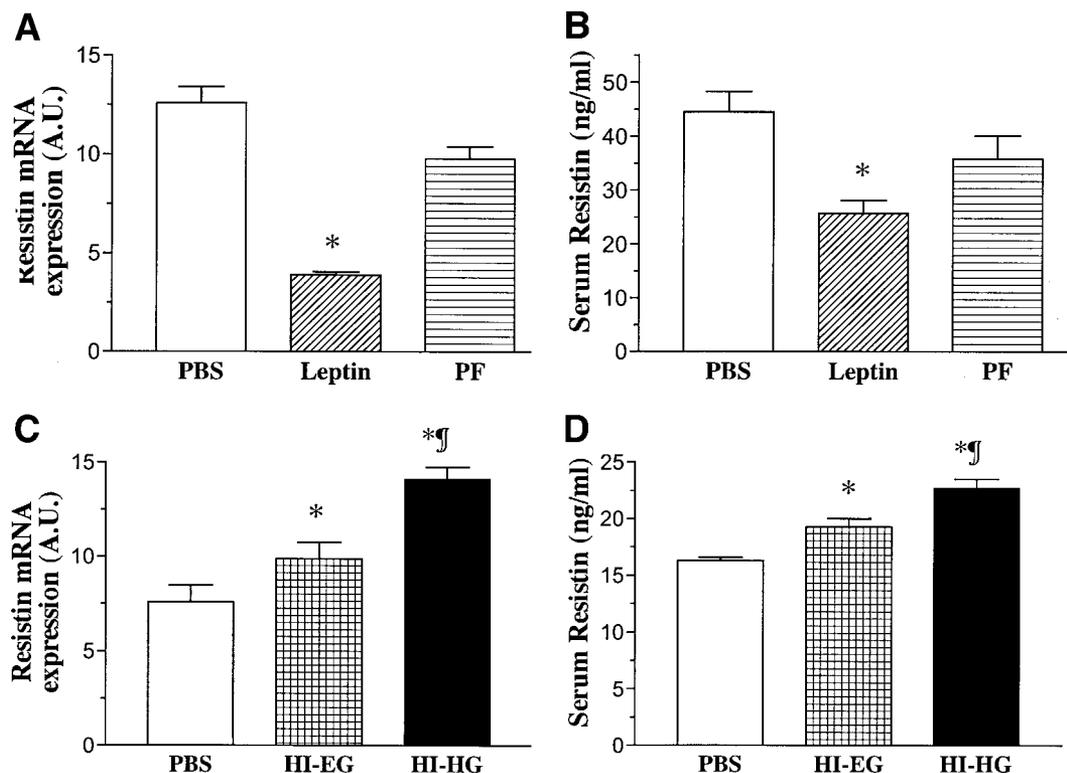


FIG. 5. Effect of leptin treatment on resistin mRNA expression in epididymal WAT (A) and serum resistin (B) levels in *Lep^{ob/ob}* mice. PF, pair-fed; $n = 6-8$. * $P < 0.05$ vs. PBS. Wild-type mice were subjected to a hyperinsulinemic-euglycemic clamp (HI-EG) or hyperinsulinemic-hyperglycemic clamp (HI-HG), resistin mRNA expression in epididymal WAT (A) and serum resistin (B) were measured. Data are means ± SE, $n = 5$. * $P < 0.05$ vs. PBS; † $P < 0.05$ vs. HI-EG.

To address the apparent discrepancy between resistin gene expression and protein levels in obese and diabetic animals, we needed a reliable method to measure resistin protein concentration in adipose tissue and serum. Here, we demonstrate that serum resistin can be reliably and accurately measured using an RIA. The specificity of the RIA was confirmed by the absence of a signal in resistin-null mice (9) and by a drastic reduction in resistin in the lipodystrophic ap2/DTx mice. We found that the diurnal resistin level was lower than the nocturnal level, and this pattern paralleled the changes in insulin and glucose. Moreover, resistin mRNA and protein levels were both suppressed after food deprivation and increased by refeeding in a pattern reminiscent of the regulation of leptin, as opposed to that of adiponectin (15,17). The fall in resistin gene expression and rise during refeeding is in agreement with a previous study in rats by Kim et al. (12). As in our study, the effect of feeding on rat resistin expression paralleled the changes in insulin and glucose (12). Importantly, we found that resistin gene expression and protein concentration in fasted mice were both increased by insulin and potentiated by glucose. This finding also supports and extends a previous report in rats (11). In that study, glucose and insulin independently increased resistin mRNA level (11). Thus, taken together, these data suggest that the nutritional regulation of resistin in rodents is mediated, at least in part, through insulin and glucose.

Another important finding of this study was the confirmation of high circulating resistin in obese mice, despite reduced mRNA level in adipose tissue. The latter confirms previous reports (10,11). Potential reasons for the high serum resistin levels in obese mice include a prolongation of protein half-life as a result of binding by serum factors and/or reduced clearance. The serum concentration could be influenced by binding proteins or tertiary changes similar to that seen for other adipokines such as leptin and tumor necrosis factor- α (25–27). It is also possible that the high circulating resistin level could suppress resistin gene expression via a negative feedback mechanism, resulting in a divergence in mRNA versus protein levels. Regardless, since resistin is secreted and thought to act as a hormone (5,9,13), we believe that studies based on associations between mRNA expression and obesity or diabetes may provide few insights and perhaps misleading conclusions about its biological role.

Despite the divergence in resistin mRNA and protein levels in *Lep^{ob/ob}* mice, both were suppressed by leptin treatment, concomitant with the reduction in glucose and insulin. In contrast, weight loss from pair-feeding (food restriction) had no significant effect on resistin and failed to decrease insulin and glucose, suggesting that the response to leptin was specifically related to improvement in insulin sensitivity. Our data are consistent with a recent study by Asensio et al. (28). In the latter study, systemic leptin treatment decreased resistin mRNA expression in *Lep^{ob/ob}* mice in parallel with glucose and insulin. Interestingly, administration of leptin into the cerebral ventricle resulted in a similar suppression of resistin gene expression in rats (28). Thus, it is possible that leptin's effect to reduce glucose involves the suppression of resistin.

Our RIA results are contrary to a report by Maebuchi et al. (29), demonstrating reduction in both resistin mRNA

and protein levels in obese mice. However, it is noteworthy that resistin protein concentration was measured in adipose tissue lysate using a "semiquantitative" ELISA. Importantly, resistin was not detected in the serum using this ELISA. Moreover, few details regarding the accuracy and precision of the assay were reported; the specificity of their assay was not verified in resistin-null mice or in lipodystrophic models (29).

In summary, we have characterized a specific and accurate RIA for mouse resistin. We have shown that both resistin gene expression and protein levels are regulated in parallel with insulin and glucose during fasting and refeeding. For the first time, we have confirmed that the nutritional regulation of resistin mRNA and protein levels in mice is likely to be mediated, at least in part, through insulin and glucose. Moreover, our data suggest that the association among resistin, glucose, and insulin in obesity may be explained by leptin's effect on insulin sensitivity. The ability of leptin to regulate resistin expression and protein concentration suggests a counterregulatory interaction between these adipokines to influence glucose homeostasis. Overall, our data are consistent with previous work suggesting a link between resistin and glucose in rodents (5,8,9). The availability of the resistin RIA will greatly facilitate efforts to better understand the underlying mechanisms.

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