METHODS

Mice. Targeting strategy for the disruption of the endogenous resistin gene locus is depicted in fig. S1A, and was performed in TL1 mouse ES cells as previously described (1). Results presented are studies of F2 and F3 mice on a mixed 129SvEv / C57Bl6 background. Age-matched male mice were used for all experiments. Genotyping was performed at weaning (21d) on genomic tail DNA. Primers (5’-TGT CGG TCA GTT GAG AAC TGA-3’) and (5’-ACT GTG CAA CAA TTC CCA CAC-3’) produced a 205 (wild type) or 317 (null) bp PCR product (fig. S1B). Animals were housed up to 5 per cage in a ventilated isolator cage system in a 12 hr light/dark cycle (lights on at 0700), with free access to water and chow or a high-fat diet (45 kcal% fat (D12451) Research Diets Inc.,New Brunswick, NJ). Weights of (+/+ ) and (-/-) mice on normal chow increased by 30.8% ± 1.9% and 30.3% ± 3.3%, respectively (p=0.9), between 6 and 16 weeks of age. In comparison, weights of (+/+ ) and (-/-) mice begun on high-fat diet at 6 weeks of age increased by 47.6 % ± 7.6% and 54.6% ± 4.4%, respectively (p=0.4) at 16 weeks of age. The weight increase between 6 and 16 weeks of age was significantly greater on high fat diet than on normal chow for both genotypes (p<0.02). All studies were approved by the University of Pennsylvania School of Medicine Institutional Animal Care and Use Committee.

X-gal staining. Fresh tissues were fixed in 0.5% glutaraldehyde for 1 h at room temperature, rinsed several times in 1XPBS and incubated in x-gal staining solution (0.1M K₃Fe(CN)₆, 0.1 M K₄Fe(CN)₆, and 1 mg/ml x-gal in 1XPBS) for 4 h at 37°C. Tissues were rinsed and fixed overnight in 10% neutral buffered formalin, prior to paraffin embedding and sectioning.
Analytical methods and measurements. Blood glucose measurements were obtained from tail vein bleeding using the OneTouch Ultra Glucometer (Lifescan; Johnson & Johnson, Milpitas, CA). Serum was harvested either by tail vein bleeding, or cardiac puncture, spun at 4ºC for 30 min and assayed immediately or stored at –80ºC. Serum resistin (Linco Research, St. Louis, MO), adiponectin (Linco Research, St. Louis, MO) and glucagon (Penn Diabetes Center) levels were measured by radioimmunoassay (RIA). Serum insulin (Rat Insulin ELISA with mouse insulin standards) and leptin (Mouse Leptin Assay ELISA) were measured with kits from Crystal Chem Inc. (Chicago, Ill). Triglyceride levels were assessed using the TG kit (Wako Chemicals USA, Richmond, VA). AMP-kinase activity was assayed using Substrate for AMPK (SAMS, Upstate Biotechnology, Lake Placid, NY) peptide as substrate (2).

Resistin administration to fasted mice. Resistin-Flag was purified as previously described (3) with the following modifications. HEK-293 cells were stably transfected with resistin-Flag subcloned into the bicistronic expression vector pIRESneo (Clontech, Palo Alto, CA) or with vector alone. Conditioned media was collected and subjected to immunopurification using Flag antibody-agarose (Sigma, St. Louis, MO), which was eluted with Flag peptide in PBS. Purification to homogeneity was confirmed by silver stain and immunoblot, and endotoxin levels measured at less than 0.008 endotoxin unit per mL using the limulus amoebocyte lysate assay. Protein concentration was determined using a mouse resistin RIA (Linco, St. Louis, MO). Rstn (-/-) mice (23 weeks of age, started on high-fat diet at age 6 weeks) were fasted for 5 hours (from 0800 to 1300) then received i.p. administration of either vehicle (mock purified conditioned media, 300 µl) or resistin (1.5 µg in 300 µl). Blood glucose was measured in a double-blind manner at the time of injection and at half hour intervals for the following 3 hours during continued fasting. Serum level of resistin, measured 2 hours after administration, was 64.6 ± 16.1 ng/ml (n = 3). For comparison, serum resistin level in (+/+)) littermates on high fat diet 2h after
Treatment with vehicle was 78.1 ± 4.9 ng/mL (n = 5, p=.0.35 vs (-/-) 2 hours after resistin injection).

**Tolerance testing.** Tolerance tests for glucose (1) and pyruvate (4) tolerance were performed on awake mice after an overnight (16 hour) fast.

**Hyperinsulinemic-euglycemic clamp studies.** Mice that had been on high fat diet from age 6 weeks were studied by hyperinsulinemic-euglycemic clamp as previously described (5), except that somatostatin was not infused. Mice were studied at 20 weeks of age in experiments shown in Fig. 2 and fig. S6, and at 18 weeks of age in experiments shown in Fig. 3B-F. Administration of resistin in saline (2.5 or 5 µg bolus then 3 µg per hour for 90 min) to rstn (-/-) mice during hyperinsulinemic-euglycemic clamp was performed essentially as described (6). Mean resistin levels by RIA were 1.1 ± 2.7 ng/ml, 61 ± 7 ng/ml, 74 ± 10 ng/ml, and 93 ± 6 ng/ml in (-/-) mice that received saline, (+/+) mice, (-/-) that received resistin with 2.5 µg bolus, and (-/-) mice that received resistin with 5 µg bolus, respectively. Results for mice bolused with 2.5 and 5 µg of resistin (mean serum level 84 ± 7 ng/ml) were combined for data analysis.

**Northern and immunoblotting.** RNA isolation and Northern blotting was performed as described(1) with hybridization to 32P labeled cDNA probes for resistin, G6Pase, and PEPCK. Radiograph images were obtained on a STORM 840 phosphoimager and quantified with ImageQuant 2.1 (Molecular Dynamics, Sunnyvale, CA). Immunoblotting was performed as described(1) using commercially available antibodies to resistin (Linco Research, St. Louis, MO); Akt, phospho-Akt (Ser473), phospho-ACC, phospho-FoxO1 (Cell Signaling, Beverly, MA); PGC1α, HNF4α, and actin (Santa Cruz Biotech., Santa Cruz, CA). Antibodies to total and phospho-AMPK were a kind gift of J. Mu and M. Birnbaum.
Statistical Analyses. Data are depicted as the mean ± the SEM. Significance of the difference between the two groups was assessed with the Student’s unpaired two-tailed t test. Correlations were analyzed with multiple regression and ANOVA. Analyses were performed using Microsoft Excel and StatView software programs.

LITERATURE CITED

Figure S1. Rstn knockout. (A) Schematic of knockout strategy. Note that LacZ containing a nuclear localization signal (NLS) replaces resistin coding exons (blue), and Neo-TK is removed by Cre transfection prior to implantation of ES cells. (B) PCR genotyping. PCR was performed on 8 mice with primers shown in Supplemental Fig. 1A "Con"=control PCR reaction in absence of DNA. (C) Genetic analysis of first 100 pups resulting from crosses of rstn (-/-) heterozygotes.
Supplemental Figure S2

Figure S2. X-gal staining of tissues from resistin null mice. (A) Kidney and perirenal white adipose tissue (WAT). (B) Liver. (C) Interscapular brown adipose tissue (BAT) and interlaced WAT. Staining of tissues from resistin heterozygous and homozygous null mice was similar.
Figure S3. Authentication of resistin null mice. (A) Northern (top) and immunoblot (bottom) analysis of tissues from wild type (WT) and resistin null mice. (B) Serum immunoblot. "*", non-specific band. (C) Radioimmunoassay of serum resistin levels. *p<0.0005. Actual values were: (+/+): 26.3 ± 1.7 ng/ml, (-/-): 1.9 ± 1.7 ng/ml. Resistin level in (+/-) mice was 20.6 ± 1.7 ng/ml [p=0.05 versus (+/+)], i.e., more than 50% of the (+/+ level). This is presumably due to compensatory changes in resistin production and/or secretion via unknown mechanisms.
**Figure S4.** Glucose tolerance test of mice 10 weeks of age after 4 weeks on a high fat diet. N=9 (+/+), and N=8 (-/-). *p<0.05.
Figure S5. Molecular phenotype of resistin null mice. (A) Gluconeogenic gene expression. Northern blot of RNA from livers of mice clamped in experiment shown in Fig. 3B. Densitometric analysis of these data, quantified by phosphorimager (Molecular Dynamics), is shown in Figs. 2B, C. n=5 (+/+), n=4 (-/-). (B) Immunoblot for indicated proteins and their modifications. (C) AMPK activation in livers of the clamped mice. n=5 (+/+), n=4 (-/-). These data are quantified using Imagequant in Figs. 2D, E.
Figure S6. Effect of Resistin Replacement on Hepatic Phospho-AMPK Levels. Livers of mice clamped in the experiment shown in Fig. 2 were analyzed by immunoblot for phospho-AMPK and total AMPK levels. * p<0.05 versus other measurements shown. No other differences were significant.
Supplemental Table 1. Weights and serum chemistries of *rstm* (+/+) and (-/-) mice. Measurements were made after a 4 hour fast of mice (14 weeks old) that had been fed a high fat diet for 8 weeks (same samples as in Fig. 1C). n=17-19 (+/+), n=15-16 (-/-), except for adiponectin [n=9 (+/+), n=8 (-/-)].

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<th>Measurement</th>
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