Loss of resistin ameliorates hyperlipidemia and hepatic steatosis in leptin-deficient mice

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THE METABOLIC SYNDROME is characterized by abdominal obesity, hypertension, dyslipidemia, and an impairment of glucose metabolism (5, 9). Studies have suggested a link between the altered production and secretion of adipokines and the pathogenesis of the metabolic syndrome (11, 21). TNF-α is increased in obesity and insulin resistance and, along with other inflammatory mediators, is thought to contribute to dyslipidemia (4). Conversely, adiponectin levels are reduced in obesity and may contribute to insulin resistance, diabetes, and dyslipidemia (14, 40).

Resistin is secreted by adipocytes in rodents and monocytes in humans (38). Peripheral resistin infusion or the transgenic overexpression of resistin impairs insulin action in rodents (26, 32, 34, 37). Conversely, genetic ablation or downregulation of the retn gene improves insulin sensitivity (3, 19, 27). Although resistin has predominantly been studied with regard to its role in impairing insulin action in rodents, increased resistin expression has also been associated with dyslipidemia and non-alcoholic fatty liver disease in some patients (21, 22). Consistent with a role for resistin in modulating lipid metabolism, resistin increases the accumulation of lipids in skeletal muscle cells by inhibiting β-oxidation (23). Resistin also promotes lipid accumulation in macrophages (30, 41). Furthermore, the adenovirus-mediated overexpression of resistin in mice increased plasma low-density lipoprotein (LDL) cholesterol, decreased high-density lipoprotein (HDL) cholesterol, and increased hepatic very-low-density lipoprotein (VLDL) production rates (33). Thus we hypothesized that the loss of the retn gene will improve lipid parameters and prevent hyperlipidemia. We examined the role of resistin in lipid metabolism by deleting the retn gene in wild-type (WT) fed a standard rodent chow or high-fat diet and leptin-deficient ob/ob mice (3, 27, 31).

MATERIALS AND METHODS

Animal studies. Experimental procedures were in accordance with protocols approved by the Animal Care and Use Committee of the University of Pennsylvania and adhered to American Physiological Society Guiding Principles in the Care and Use of Animals. Heterozygote resistin-deficient mice (+/−) on the original 129/C57Bl/6J mixed background (3) were backcrossed onto C57Bl/6J background (Jackson Laboratories, Bar Harbor, ME) for nine generations, followed by heterozygote matings to produce WT and resistin-knockout (RKO) mice (27). To generate mice deficient in resistin and leptin, retn (+/−) mice were mated with C57Bl/6J leptin−/− (−/−) mice (Jackson Laboratories), and double heterozygotes were then mated to generate ob/ob, ob/ob-RKO, RKO, and WT (27). The mice were weaned at 3 wk and housed (n = 5/cage) in 12-h:12-h light-dark cycle (lights on 7:00) at an ambient temperature of 22°C. Normal chow (Lab Diet 5001; Richmond, IN) and water were provided ad libitum to one group of mice. Another group was fed a high-fat (45%) diet (No. D12451; Research Diets, New Brunswick, NJ) for 10 wk (31). Body composition was measured using dual emission X-ray absorptiometry (DEXA) as previously described (27). To assess VLDL secretion, 14-wk-old male mice were fasted for 4 h (8:00–12:00 h) and received 1 g/kg poloxamer-407 (intraperitoneally) (13, 18). Tail blood was drawn at time 0 and 1 and 4 h later, and serum was prepared for triglyceride measurement (13, 18, 39). The detergent poloxamer-407 inhibits lipases and prevents the clearance of triglycerides, thereby allowing for the assessment of its production (13, 18).

Tissue chemistry. At 14 wk of age, the mice were euthanized following a 4-h fast using CO2 inhalation between 12:00 and 13:00 h, and blood was obtained via cardiac puncture. Cohorts of mice used for the analysis of serum chemistries and gene expression were different from those used for VLDL secretion. Samples of the liver were rapidly excised, frozen in liquid nitrogen, and stored at −80°C. Serum triglycerides, cholesterol, nonesterified fatty acids (NEFA), and β-hydroxybutyric acid were measured using enzymatic assays (Stanbio Labs and Wako Chemicals) (27, 28). Pooled serum samples (125 μl) from each group were analyzed by fast-performance liquid chromato- graphy (FPLC; Amersham Pharmacia Biotech, Uppsala, Sweden) as described (18, 39). Lipids were extracted from the livers for measurement of triglycerides and cholesterol (13, 18, 39). Serum levels of the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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insulin, resistin, leptin, and adiponectin were measured using enzyme immunoassays (3, 27, 28).

**Gene expression.** RNA was extracted from the livers using TRIzol reagent (Invitrogen, Carlsbad, CA). Following treatment with DNase I, the RNA was reverse transcribed with SuperScript Reverse Transcriptase (Invitrogen) and amplified using Taqman Universal PCR Master Mix with Taqman Assay-on-Demand kits (Applied Biosystems). Quantitative RT-PCR was performed using an ABI-Prism 7800

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**Fig. 1.** Effects of resistin deficiency on body weight (A and B), fat (C), lipids (D–F), ketones (G), glucose (H), insulin (I), resistin (J), leptin (K), and adiponectin (L). Wild-type (WT) and resistin-knockout (RKO) mice were fed a high-fat diet for 10 wk, and ob/ob and ob/ob-RKO mice were fed normal chow. Data are means ± SE (n = 5/group). *P < 0.01 vs. WT; **P < 0.001 vs. WT; †P < 0.01 vs. ob/ob. Dotted line denotes level in WT mice on normal chow. NEFA, nonesterified fatty acids.
sequence detector (Applied Biosystems) (3, 13, 39). The expression of mRNA levels of lipogenic and lipolytic genes was normalized to 36B4 (3, 13, 39).

**Immunoblotting.** Liver samples were homogenized in lysis buffer containing 50 mM Tris·HCl (pH 7.4), 250 mM mannitol, 0.5% (wt/vol) Triton X-100, 50 mM NaF, 1 mM sodium pyrophosphate, 1 mM benzamidine, and 1 mM phenylmethanesulfonyl fluoride supplemented with a complete protein inhibition cocktail tablet from Roche (Penzberg, Germany). Protein extracts were separated by 4–12% NuPAGE Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membranes using semidy transfer cells (Bio-Rad, Hercules, CA). After 1 h of blocking with Tris-buffered saline with 0.1% (vol/vol) Tween 20 (TBST) containing 3% (wt/vol) nonfat dried milk, membranes were incubated with a polyclonal antibody against acetyl-CoA carboxylase (ACC; Millipore), fatty acid synthase (FAS; Cell Signaling), stearoyl-CoA desaturase-1 (SCD1; Cell Signaling), 3-hydroxy-3-methyl-glutaryl-CoA-reductase (HMGCoAR; Cell Signaling), or glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz) at 1:1,000 concentration overnight at 4°C, washed three times with TBST, incubated with horseradish peroxidase-conjugated goat anti-guinea pig IgG (Santa Cruz Biotechnology) for 1 h at room temperature, and visualized with enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). Film autoradiograms were analyzed using laser densitometry and ImageJ (National Institutes of Health). Protein lysates from the livers of WT, ob/ob, and resistin-deficient mice were also blotted for AMP-activated protein kinase (AMPK), phosphorylated (p)-AMPK, suppressors of cytokine signaling (SOCS)-3, and β-actin as we have previously described (3, 27, 32).

**Liver histology.** Liver samples were fixed in 10% buffered formalin overnight, embedded in paraffin, sectioned, and processed for hematoxylin-eosin staining (13, 39). The slides were examined under brightfield microscopy (Nikon E600), and images were captured using a Cool Snap CF digital camera (BD Biosciences Bioimaging, Rockville, MD).

**Statistics.** Data are presented as means ± SE. The effects of retn deletion in diet-induced obese (DIO) and ob/ob mice were analyzed by ANOVA, and differences between groups were assessed by Fisher's
paired least significant difference test (GraphPad Prism, San Diego, CA). \( P < 0.05 \) was considered significant.

RESULTS

Resistin deficiency decreases hepatic triglyceride secretion and cholesterol in DIO C57Bl/6J mice. RKO mice exhibited no differences in body weight, adiposity, or serum chemistry compared with those of WT mice on standard rodent chow, in agreement with our previous reports (3, 27). However, hepatic triglyceride content showed a tendency to decrease in chow-fed RKO mice (7.4 ± 1.04 mg/g in RKO vs. 10.5 ± 1.2 mg/g in WT, \( P = 0.101 \)). Thus we studied the effects of resistin in DIO mice (Fig. 1, A–L). The high-fat diet increased body weight and fat and serum lipids, glucose, insulin, resistin, and leptin (Fig. 1, A, C, D, I, J, and K), whereas NEFA, \( \beta \)-hydroxybutyric acid, and adiponectin were not altered (Fig. 1, F, G, and L). Resistin deficiency did not affect body weight (Fig. 1A) or total fat content (Fig. 1C) or abdominal fat measured by DEXA (data not shown) in DIO mice. Nonetheless, the hepatic content of triglyceride and not cholesterol was reduced in the absence of resistin (Fig. 2, A and B). Hepatic steatosis was attenuated in DIO RKO mice (Fig. 2, C and D), and the VLDL secretion and HDL fraction of cholesterol were also suppressed (Fig. 2, E and F).

Resistin deficiency increases obesity but reduces hepatic steatosis and lipid levels in ob/ob mice. Leptin deficiency results in early onset obesity, hyperinsulinemia, steatosis, and hyperlipidemia (Figs. 1 and 3) (1, 10, 24, 27, 39). As we have previously reported, \( ob/ob \) mice lacking resistin were more obese (Fig. 1, A–C) (27). Despite a higher body weight and...
Adiposity, the serum levels of triglycerides, cholesterol, glucose, and insulin were lower in ob/ob-RKO mice (Fig. 1, D, E, H, and I). Triglyceride content in the liver was also drastically reduced in ob/ob-RKO mice (Fig. 2A) in parallel with hepatic steatosis (Fig. 3B). Moreover, resistin deficiency restored normal histology in periporal hepatocytes (Fig. 3, C and D). The secretion of VLDL from the liver (Fig. 3E) and intermediate density lipoprotein (IDL) and HDL cholesterol fractions assessed by FPLC (Fig. 3F) were also decreased in ob/ob-RKO mice.

Resistin deficiency suppresses lipogenic gene expression. We analyzed the effects of resistin on pathways involved in hepatic lipid metabolism (13, 28, 39). The mRNA levels of sterol regulatory element binding protein (SREBP)-1, ACC, FAS, SCD1, diacylglycerol O-acyltransferase (DGAT)-2, apoB, microsomal triglyceride transfer protein (MTTP), and HMGCoAR were increased in ob/ob and fell in resistin-deficient ob/ob-RKO mice (Fig. 4, A–H). Likewise, the mRNA levels of ACC, FAS, apoB, MTTP, and HMGCoAR were suppressed in the livers of resistin-deficient DIO mice (Fig. 4, B, C, and F–H). In contrast, the expression of peroxisome proliferator-activated receptor-α and carnitine palmitoyltransferase-1a was not altered by the lack of resistin in ob/ob or DIO mice (data not shown). The results of the immunoblotting of liver lysates paralleled the changes in mRNA expression. The protein levels of ACC, FAS, SCD1, and HMGCoAR were higher in ob/ob than in DIO mice (Fig. 5, A–E). Resistin deficiency decreased ACC, FAS, and SCD1 levels in both ob/ob and DIO mice (Fig. 5, A–D).

The resistin receptor is unknown, but the ability of resistin to induce insulin resistance under hyperinsulinemic clamp has been associated with the reduced phosphorylation of AMPK and the induction of SOCS-3 (3, 19, 27). However, in the absence of exogenous insulin in the current study, retn deletion did not alter hepatic levels of SOCS-3 and AMPK or the phosphorylation of AMPK (Fig. 6, A–C).

**Fig. 4.** Effects of resistin deficiency on hepatic gene expression (A–F). WT and RKO mice were fed a high-fat diet for 10 wk, and ob/ob and ob/ob-RKO mice were fed normal chow. Data are means ± SE (n = 5/group). *P < 0.05 vs. WT; **P < 0.01 vs. WT; ***P < 0.001 vs. WT; †P < 0.001 vs. ob/ob. SREBP, sterol regulated element binding protein; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD, stearoyl-CoA desaturase; DGAT, diacylglycerol O-acyltransferase; apoB, apolipoprotein B; MTTP, microsomal triglyceride transfer protein; HMGCoAR, 3-hydroxy-3-methyl-glutaryl-CoA-reductase.
DISCUSSION

We have previously demonstrated that WT mice exhibit similar body weight as those lacking resistin (3, 27). However, insulin sensitivity is improved in resistin deficiency, resulting in the suppression of hepatic glucose production and increased glucose uptake in peripheral tissues (3, 27). The deletion of retn exacerbates obesity in ob/ob mice by further lowering energy expenditure (27). Studies indicate that resistin decreases body fat by inhibiting adipogenesis (15, 16). In the current study, we compared the effects of resistin deficiency on lipids in DIO and ob/ob mice. In both models, the ablation of retn decreased triglyceride production and serum cholesterol levels and attenuated hepatic steatosis. These effects were more pronounced in ob/ob mice, suggesting that deficiencies of both leptin and resistin have major consequences on lipids. Key lipogenic genes, i.e., SREBP-1, FAS, SCD1, DGAT2, and HMGCoAR, and those involved in VLDL packaging and export, i.e., MTTP and apoB, were suppressed in the livers of resistin-deficient mice. The increased expression of SREBP-1 induces hepatic steatosis and hyperlipidemia, whereas DGAT2 suppression prevents steatosis (5, 8, 36). A decrease in the MTTP level reduces VLDL secretion (29). HDL, which is the predominant lipoprotein in mice, was reduced in parallel with HMGCoAR mRNA levels in DIO and ob/ob mice. On the other hand, the expression of genes involved in lipolysis was not affected by the lack of resistin. Together, these results suggest that resistin affects lipids mainly by regulating lipogenic enzymes.

Our findings are consistent with those of Sato et al. (33). In the latter study, the adenovirus-mediated overexpression of resistin increased triglyceride and cholesterol levels. VLDL secretion was increased by resistin, although the expression of MTTP and apoB did not change (33). Hyperinsulinemia has been associated with steatosis and hyperlipidemia in humans and animal models (2, 5, 20, 36). Conversely, treatment with insulin-sensitizing drugs ameliorates steatosis and hyperlipidemia (5, 6, 7, 10, 12, 29, 39). In the current study, the degree of steatosis was related to insulin levels in DIO WT (5.14 ± 0.4
Obesity has been related to the inhibition of AMPK and insulin sensitivity (32, 34). Recombinant resistin or transgenic overexpression impaired glucose tolerance (19). Conversely, an increase in resistin via an infusion of antisense oligonucleotide treatment increased insulin sensitivity (18). Likewise, a reduction in the circulating resistin level via leptin-deficient ob/ob mice (3, 27). We have reported that the ablation of adiponectin interact to produce zonal patterns of steatosis. Insulin and glucose levels were reduced in ob/ob-RKO compared with ob/ob mice, suggesting these factors may play a role in determining the pattern of steatosis. Adiponectin is thought to be protective against steatosis in rodents and humans (14, 40). However, the levels of adiponectin did not change in DIO and ob/ob mice lacking resistin, arguing against a significant role of adiponectin in mediating the lipid dysregulation in these particular models (14, 40).

In summary, our studies demonstrate that resistin is an important mediator of hepatic steatosis and hyperlipidemia, particularly in mice lacking leptin. A further examination of the interactions between these adipokines may provide valuable insight into the pathogenesis and treatment of lipid abnormalities associated with obesity and the metabolic syndrome.

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Histological abnormalities associated with nonalcoholic fatty liver disease mainly affect pericentral hepatocytes (12). Leptin-deficient ob/ob mice exhibited steatosis in all zones of the hepatic lobule. In contrast, pericentral hepatocytes were mildly steatotic, whereas periportal hepatocytes were spared in ob/ob-RKO mice. These findings suggest that leptin and resistin interact to produce zonal patterns of steatosis. Insulin and glucose levels were reduced in ob/ob-RKO compared with ob/ob mice, suggesting these factors may play a role in determining the pattern of steatosis. Adiponectin is thought to be protective against steatosis in rodents and humans (14, 40). However, the levels of adiponectin did not change in DIO and ob/ob mice lacking resistin, arguing against a significant role of adiponectin in mediating the lipid dysregulation in these particular models (14, 40).

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Fig. 6. Lack of effect of resistin deficiency on putative mediators of hepatic insulin resistance. WT and RKO mice were fed a high-fat diet for 10 wk, and ob/ob and ob/ob-RKO mice were fed normal chow (A–C). Data are means ± SE arbitrary densitometric units. Suppressors of cytokine signaling (SOCS)-3 was normalized to β-actin, and phosphorylated (p)-AMP-activated protein kinase (AMPK) was normalized to total AMPK (n = 3/group).


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