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Resistin and Type 2 Diabetes: Regulation of Resistin Expression by Insulin and Rosiglitazone and the Effects of Recombinant Resistin on Lipid and Glucose Metabolism in Human Differentiated Adipocytes

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Resistin, an adipocyte secreted factor, has been suggested to link obesity with type 2 diabetes in rodent models, but its relevance to human diabetes remains uncertain. Although previous studies have suggested a role for this adipocytokine as a pathogenic factor, its functional effects, regulation by insulin, and alteration of serum resistin concentration by diabetes status remain to be elucidated. Therefore, the aims of this study were to analyze serum resistin concentrations in type 2 diabetic subjects; to determine the *in vitro* effects of insulin and rosiglitazone (RSG) on the regulation of resistin, and to examine the functional effects of recombinant human resistin on glucose and lipid metabolism *in vitro*. Serum concentrations of resistin were analyzed in 45 type 2 diabetic subjects and 34 nondiabetic subjects. Subcutaneous human adipocytes were incubated *in vitro* with insulin, RSG, and insulin in combination with RSG to examine effects on resistin secretion. Serum resistin was increased by approximately 20% in type 2 diabetic subjects compared with nondiabetic subjects ($P = 0.004$) correlating with C-reactive protein. No other parameters, including adiposity and fasting insulin levels, correlated with serum resistin in this cohort. However, *in vitro*, insulin stimulated resistin protein secretion in a concentration-dependent manner in adipocytes [control, 1215 ± 87 pg/ml (mean ± SEM); 1 nM insulin, 1414.0 ± 89 pg/ml; 1 μM insulin, 1797 ± 107 pg/ml ($P < 0.001$)]. RSG (10 nM) reduced the insulin-mediated rise in resistin protein secretion (1 nM insulin plus RSG, 971 ± 35 pg/ml; insulin, 1 μM insulin plus RSG, 1019 ± 28 pg/ml; $P < 0.01$ *vs.* insulin alone). Glucose uptake was reduced after treatment with 10 ng/ml recombinant resistin and higher concentrations ($P < 0.05$). Our *in vitro* studies demonstrated a small, but significant, reduction in glucose uptake with human recombinant resistin in differentiated preadipocytes. In human abdominal sc adipocytes, RSG blocks the insulin-mediated release of resistin secretion *in vitro*. In conclusion, elevated serum resistin in human diabetes reflects the subclinical inflammation prevalent in type 2 diabetes. Our *in vitro* studies suggest a modest effect of resistin in reducing glucose uptake, and suppression of resistin expression may contribute to the insulin-sensitizing and glucose-lowering actions of the thiazolidinediones. (*J Clin Endocrinol Metab* 88: 6098–6106, 2003)

RESISTIN (FIZZ3) BELONGS to a family of cysteine-rich, C-terminal proteins known as resistin-like molecules (RELM; RELM α/FIZZ 1 and RELMβ/FIZZ 2) of FIZZ (found in inflammatory zone) that are believed to be involved in inflammatory processes (1–3). Previous studies have shown that in mice, resistin impairs glucose tolerance and insulin action and inhibits adipogenesis in murine 3T3-L1 cells (1, 4–7). Therefore, resistin has also been proposed as an adipocyte secreted factor that is thought to link obesity and type 2 diabetes (2). The role of resistin in human obesity remains controversial. Previous resistin mRNA expression studies have produced conflicting reports about the level of expression of this gene in human adipose tissue (8–11). However, recent studies also indicate higher levels of expression of both gene and protein in central adipose tissue depots, supporting a role for resistin in linking central obesity to diabetes (12).

Thiazolidinediones (TZD) are a class of antidiabetic drugs that enhance target tissue sensitivity to insulin (4). Lazar and co-workers (1) were the first to demonstrate that both resistin mRNA and protein were down-regulated by TZDs *in vitro*, with the majority of subsequent *in vivo* data supporting these observations (13–15). Furthermore, in their experiments, recombinant resistin induced insulin resistance both *in vivo* and *in vitro*, an effect that was blocked by antiresistin antibodies. These data suggested a potential mechanism for obesity-related insulin resistance and also indicated a new mechanism of action for TZDs. These findings support the hypothesis that resistin may be a protein that limits obesity at the expense of glucose tolerance, at least in rodent models.

Although resistin appeared to be a potential candidate protein linking excess adiposity and insulin resistance in rodent models, many human studies described low levels of resistin mRNA expression in adipose tissue and concluded that this molecule is unlikely to be an important factor linking

Abbreviations: BMI, Body mass index; FIZZ, found in inflammatory zone; HbA_{1c}, hemoglobin A_{1c}; HOMA, homeostasis model assessment; LPS, lipopolysaccharide; RELM, resistin-like molecules; RSG, rosiglitazone; TZD, thiazolidinedione.

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human obesity to insulin resistance (8–10). Our previous studies have, however, shown that resistin mRNA and protein expression are significantly increased in human central adipose tissue depots (both omental and sc) compared with thigh and breast adipose tissue (11, 12). In addition, we confirmed increasing resistin mRNA expression with obesity, in agreement with previous findings by Savage *et al.*, (8). These findings were not related to the contamination of adipose tissue samples by mononuclear blood cells, which are known to express resistin (8, 9, 12).

It is clear that further studies are needed to understand the role of resistin in human obesity and type 2 diabetes. Although our previous human resistin mRNA and protein expression adipose tissue studies have implicated this adipocytokine as a potential pathogenic factor in central adiposity, its functional effects on glucose and lipid metabolism and its regulatory role by insulin remain unknown. The present study therefore examined whether resistin was simply a marker of diabetes status or was a regulated and functionally important cytokine that potentially contributed to the pathogenesis of obesity-related type 2 diabetes. We also examined the relationship of resistin to subclinical inflammation in view of studies demonstrating the expression of resistin in macrophages (8, 9). Therefore, to extend our investigations we validated a commercially available ELISA and then assessed serum concentrations of resistin in nondiabetic and type 2 diabetic subjects. Serum resistin was also assessed for correlations with other clinical and biochemical parameters, including fasting serum glucose, insulin, leptin, hemoglobin A_{1c} (HbA_{1c}), and C-reactive protein concentrations as well as anthropometric data. Next, we examined the *in vitro* effects of insulin and TZDs on resistin expression in isolated human adipocytes. Finally, we examined the effect of recombinant human resistin on glucose uptake, lipolysis, and lipogenesis in human differentiated preadipocytes.

Subjects and Methods

Subjects

Serum resistin levels were examined in 45 nondiabetic subjects [9 women and 36 men; age, 40.2 ± 12.8 yr (mean ± SD); body mass index (BMI), 33.04 ± 10.64 kg/m^2] and 34 type 2 diabetic subjects (5 women and 29 men; age, 54.3 ± 13.03 yr; BMI, 30.5 ± 6.3 kg/m^2). Subcutaneous abdominal adipose tissue was obtained from a human population (female/male ratio, 15:3; age, 43.7 ± 5.2 yr; BMI, 25.3 ± 2.7 kg/m^2). Subjects providing fat samples were not receiving endocrine therapy (*e.g.* steroids, hormone replacement therapy, or T_4) or any antihypertensive therapy and were not diabetic. All studies were carried out with the approval of the local ethics committee, and patients provided informed consent.

Collection and analysis of serum samples

For the collection of blood, samples were taken after a 12-h fast for the determination of serum glucose, insulin, leptin, resistin, HbA_{1c}, and C-reactive protein concentrations. At the same time, all anthropometric data were collected. Specific measurement of serum HbA_{1c} was performed using routine laboratory procedures. Insulin was analyzed by a solid phase enzyme-amplified sensitivity immunoassay (Medgenix Ins-ELISA, BioSource, Nivelles, Belgium). Insulin resistance [determined by homeostasis model assessment (HOMA)] and β-cell function were derived using the HOMA equation (16). C-Reactive protein was measured using a particle-enhanced immunological assay (Roche, Basel, Switzerland).

Isolation and cell culture of mature adipocytes

Subcutaneous abdominal adipose tissue was digested as previously described to isolate mature adipocytes and preadipocytes (17). After isolation of these cell types, mature adipocytes (n = 14) were cultured in phenol red-free DMEM/Ham's F-12 medium containing 15 mmol/liter glucose, penicillin (100 U/ml), and streptomycin (100 μg/ml). Compacted (1-ml) aliquots of adipocytes (500,000 cells) were maintained in medium (5 ml) for 48 h and treated with insulin alone (1–1 μM), RSG alone (0.1 nM to 10 μM; GlaxoSmithKline, Harlow, UK), or insulin (1–1 μM) in combination with RSG (10 nM). Additionally, a time-course study was undertaken to assess changes in mRNA and protein secretion of resistin at 4, 12, and 24 h with control and insulin-treated adipocytes (1 and 100 nM). Adipocytes maintained in untreated medium for 48 h were used as the controls. The viability of adipocytes was assessed using the trypan blue dye exclusion method as previously documented (Sigma-Aldrich Corp., Poole, UK) (16). After treatment, the medium and adipocytes were separated by centrifugation (360 × g for 2 min). The medium was removed, aliquoted, and stored at −70 C.

Differentiation of preadipocytes

Abdominal sc preadipocyte cells (n = 7) were grown in 12-well plates to confluence in DMEM/Ham's F-12 medium containing 15% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and transferrin (5 μg/ml). At confluence, preadipocytes were differentiated in DMEM/Ham's F-12 phenol-free medium containing glucose (1000 mg/liter), penicillin (100 U/ml), streptomycin (100 μg/ml), transferrin (5 μg/ml), T_3 (0.2 nM), panthenoate (17 μmol/liter), biotin (33 μmol/ml), insulin (100 nM), and cyclodextran-coated cortisol (100 nM) (18, 19). This chemically defined, serum-free medium was changed every 2 d for 12 d. Cells were also treated with 1-methyl-3-isobutylxanthine during the first 4 d of differentiation as previously described (19). Cells were treated in triplicate with varying concentrations of recombinant resistin (Research Diagnostics, Flanders, NJ; 0.1, 1, 10, 30, and 50 ng/well) during differentiation of the preadipocytes. Control was taken as preadipocytes maintained in differentiation medium without recombinant resistin treatments for the duration of the study. Conditioned media from the differentiating preadipocytes were collected every other day for the duration of the 12 d and stored at −70 C. Additionally, sc preadipocytes were differentiated until d 11 in chemically defined medium alone and subsequently treated with recombinant resistin (30 ng) for 24 h.

Western blot analysis

Homogenized human adipose tissue and adipose cells were resuspended in 4% sodium dodecyl sulfate as previously described (17). In addition, a human lung carcinoma cell line (ECACC 86012804) and human mucosal colonic tissue were used as positive controls for RELM α/FIZZ 1 and RELMβ/FIZZ 2, respectively. Western blot analysis was performed using a method previously described (20). In brief, 10–60 μg protein were loaded onto a 4–20% precast gel (Bio-Rad, Poole, Dorset, UK), and human RELM α/FIZZ 1 and RELMβ/FIZZ 2 polyclonal primary antibodies were used (1:500 and 1/250, respectively; Alpha Diagnostics, Princeton, NJ). Both RELMα/FIZZ 1 and RELMβ/FIZZ 2 were developed using antirabbit horseradish peroxidase secondary antibodies (Biogenesis, Poole, UK). Equal protein loading for these samples was confirmed by examining actin expression using Western blotting, as previously described (21). A chemiluminescent detection system (ECL/ECL$^+$, Amersham Pharmacia Biotech, Little Chalfont, UK) enabled visualization after exposure to x-ray film.

Protein assay

Protein concentrations were determined using the Bio-Rad DC (detergent-compatible) protein assay kit (22). Adipocyte protein samples were also assessed to determine that there was no statistically significant variation between the control and treatment regimens, thus indicating that the changes observed in the secretion of resistin were not due to variations in protein between samples.

Serum resistin concentrations

Serum samples were analyzed for resistin in nondiabetic (mean age, 40.2 ± 12.8 yr; mean BMI, 33.0 ± 10.6 kg/m^2) and type 2 diabetic samples

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(mean age, 54.3 ± 13 yr; mean BMI, 30.5 ± 6.3 kg/m^2) using the human resistin ELISA assay from Phoenix Pharmaceuticals (San Francisco, CA). The measurement of resistin secreted from abdominal sc adipocytes in conditioned medium samples was made after dilution with assay buffer before analysis. Due to the theoretical possibility that cross-reactivity with other RELMs may occur, we assessed cross-reaction using RELMα/FIZZ 1 and RELMβ/FIZZ 2 partial peptides (Alpha Diagnostics Eastleigh, Hampshire, UK). Known quantities of recombinant resistin (Research Diagnostics) were also used to determine recovery within the assay, using assay buffer as well as serum coincubated with RELMα/FIZZ 1 and RELMβ/FIZZ 2 partial peptides (α Diagnostics).

Incorporation of glucose into differentiated preadipocytes

For assessment of glucose uptake, a modified method of Dole and Meinertz was used (23). The differentiated preadipocytes were incubated with and without treatments, ^{14}C-labeled glucose (Amersham Pharmacia Biotech) and unlabeled glucose (5 mmol/liter) with a concentration of 0.5 mmol/liter in the chemically defined medium on the final day of incubation. After 14 h at 37 C, the medium was removed, and preadipocytes were extracted in 2% sodium dodecyl sulfate containing 25 mM Tris-HCl (pH 6.8; a 5-μl aliquot was taken for protein assay). The amount of labeled ^{14}C released from the cells as CO_2 was also measured. Filter disks (grade 1M chromatography paper, 20 mm diameter, Whatman, Cambridge, UK) presoaked in sodium hydroxide (1 M) to absorb CO_2 were attached above each well of the culture plates. These filters remained attached during the period of the experiment. Subsequently, the extracted preadipocytes and filter disks were placed in scintillation fluid (Opti-Phase 3, Wallac, Milton Keynes, UK), and the radioactivity was determined using a liquid scintillation counter.

Lipolysis studies in differentiated preadipocytes

Conditioned media collected during the course of differentiation of the preadipocytes were assessed for glycerol production as a measure of lipolysis (micromoles per milliliter) using a commercially available colorimetric kit (Randox Laboratories, Co. Antrim, UK).

Leptin secretion in differentiating preadipocytes

Conditioned medium samples collected during the course of differentiation were assayed for leptin secretion, as a marker of differentiation, using an RIA kit (Linco Research, Inc., St. Charles, MO). In addition, serum leptin analysis was performed in the nondiabetic and type 2 diabetic subjects.

Lipid staining of differentiated preadipocytes

Lipid staining was performed using a modified method of Culling *et al.* (24). Briefly, on d 12 differentiated preadipocytes were washed with Hanks' Balanced Salt Solution and stained with 2.5% Oil Red O (Gurr Ltd., London, UK) for 15 min at room temperature. After brief treatment with 60% isopropanol (Fisher Scientific Ltd., Loughborough, UK) at room temperature, cells were washed with distilled water and viewed with a light microscope. Differentiation of preadipocytes was determined by photographic assessment of the accumulation of lipid over time.

Extraction of adipocyte RNA and quantitative RT-PCR

RNA was extracted from treated isolated adipocytes using the GenElute total mammalian RNA kit (Sigma-Aldrich Corp.), which included a deoxyribonuclease digestion step to remove any contaminating genomic DNA. One microgram of RNA from each sample was reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega, Southhampton, UK) and random hexamers in 20-μl reaction volumes according to the manufacturer's instructions.

mRNA levels were determined using an ABI 7700 real-time PCR Sequence Detection system (PE Applied Biosystems, Foster City, CA), as previously described (25). The reactions were performed in 25-μl volumes on 96-well plates in reaction buffer containing TaqMan Universal PCR Master Mix (PE Applied Biosystems), 3 mM Mn(Oac)$_2$, 200 μM deoxy-NTPs, 1.25 U AmpliTaq Gold polymerase (PE Applied Biosystems), 1.25 U AmpErase UNG (PE Applied Biosystems), 150 nmol TaqMan probe, 900 nmol primers, and 50 ng cDNA (except for resistin assay for which 115 ng cDNA were used). Quantitative primer and probe sequences for resistin and CD45 genes were analyzed using previously detailed sequences (11, 12). All reactions were multiplexed with the housekeeping gene 18S, provided as a preoptimized control probe (PE Applied Biosystems), enabling data to be expressed in relation to an internal reference to allow for differences in RT efficiency. Data were obtained as Ct values according to the manufacturer's guidelines (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and were used to determine ΔCt values (ΔCt = Ct of the target gene minus Ct of the housekeeping gene). Measurements were carried out on at least three occasions for each sample. To exclude potential bias due to averaging data that had been transformed through the equation $2^{-\Delta\Delta Ct}$, all statistics were performed at the ΔCt stage.

Statistical analysis

For analysis of serum resistin and gene expression data, statistical analysis was undertaken using unpaired *t* tests unless otherwise stated, where data were analyzed using nonparametric tests. Protein expression data for control and treatment regimens were compared using ANOVA. The threshold for significance was $P < 0.05$. The data in the text and figures are presented as the mean ± SEM unless otherwise stated.

Results

Resistin assay validation: recovery of recombinant resistin

Recombinant resistin was used to generate a standard curve with the resistin ELISA from Phoenix Pharmaceuticals and produced a corresponding curve, as documented previously with the resistin peptide standards provided in the kit. Known concentrations of recombinant resistin (5, 10, 12, and 15 ng/ml) were also added to pooled serum (29.9 ng/ml) to determine whether the expected concentration correlated closely with the actual observed value. In serum, the recovery of spiked resistin was between 90–98% efficient (expected value for pooled serum coincubated with 10 ng/ml recombinant resistin, 39.9 ng/ml; actual observed values, 38.35 ± 1.2 ng/ml).

Resistin assay validation: cross-reactivity with RELMs

Known concentrations of RELMα partial peptide (12, 15, and 40 ng/ml) and recombinant resistin (25 ng/ml) were

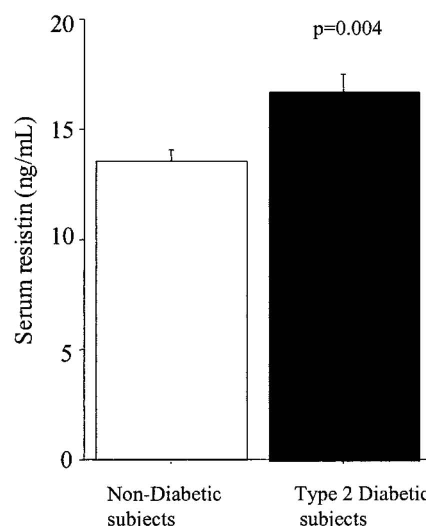

FIG. 1. Serum resistin concentrations in nondiabetic and type 2 diabetic subjects.

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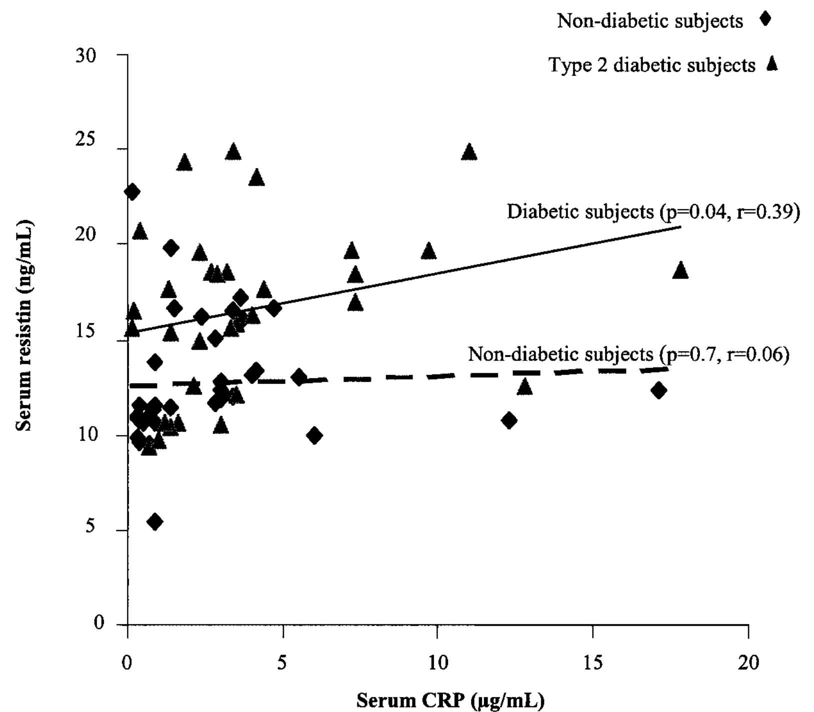

FIG. 2. Correlation of serum C-reactive protein (CRP) with serum resistin in nondiabetic and type 2 diabetic subjects. The line of best fit is drawn to examine the correlation in both cohorts.

added to an aqueous solution. However, the addition of RELMα did not interfere with the resistin ELISA assay (Phoenix Pharmaceuticals). Similar findings were observed for the RELMβ partial peptide at the same concentrations (data not shown). When RELMα and RELMβ peptides were added to pooled serum samples, these findings showed that neither peptide altered the known serum concentrations of resistin measured in the absence of peptides in the resistin ELISA. Again, the addition of RELMα and RELMβ peptides to a serum matrix containing recombinant resistin (25 ng/ml) did not affect the expected resistin concentration (data not shown).

Comparison of serum resistin in diabetic with nondiabetic subjects

Comparison of fasting serum resistin in type 2 diabetic and nondiabetic subjects showed that type 2 diabetic patients had higher resistin levels than nondiabetic subjects [diabetic subjects, 16.6 ± 0.89 ng/ml (mean ± SEM); nondiabetic subjects, 13.5 ± 0.5 ng/ml; $P = 0.004$; Fig. 1]. Additionally, we performed stepwise multiple regression analysis using resistin as the dependent value with independent values as follows: C-reactive protein, insulin, BMI, waist circumference, diabetic status, leptin, and insulin resistance using HOMA (26). This analysis determined that C-reactive protein was the only significant predictor of resistin using a Spearman correlation (Fig. 2; $P = 0.04$; r = 0.39). There was no significant ethnic difference in serum resistin levels between the Caucasian and Indo-Asian subjects with or without type 2 diabetes. No gender differences were noted in serum resistin levels. Serum leptin increased with adiposity in the nondiabetic subjects (Fig. 3). Serum leptin levels were not significantly altered by diabetic status.

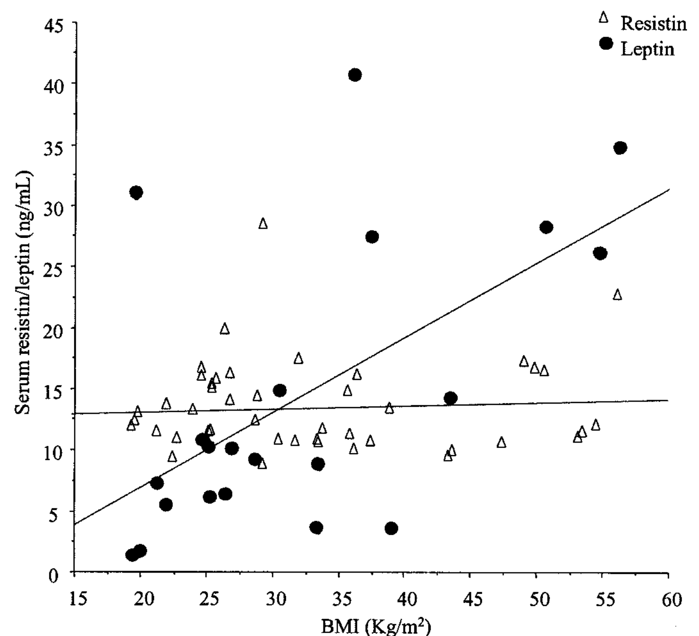

FIG. 3. Serum resistin (nanograms per milliliter) and leptin (nanograms per milliliter) levels in nondiabetic subjects and diabetic subjects were correlated with their corresponding BMIs. The line of best fit was calculated, and the correlation coefficient values (r^2) are given for each analysis (resistin: $r^2 = 0.005$; NS; leptin: $r^2 = 0.338$; $P = 0.0057$).

RT-PCR analysis of resistin expression: effects of insulin and RSG

In cultured mature human adipocytes, exposure to insulin concentrations up to 1 μM had no effect on resistin mRNA expression [control ΔCt, 20.69 ± 0.31 (mean ± SE); 100 nM

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insulin ΔCt, 20.49 ± 0.52; 1 μM insulin ΔCt, 20.61 ± 0.34; Table 1]. Furthermore, resistin mRNA expression was unaltered by RSG alone (10 nM RSG ΔCt, 20.43 ± 0.55) or insulin in combination with RSG (100 nM insulin plus 10 nM RSG ΔCt, 20.53 ± 0.36; Table 1).

Resistin mRNA expression has been reported in peripheral blood mononuclear cells (8, 12). CD45, a monocyte-specific marker, was therefore used to assess contamination of adipocyte cultures with peripheral blood mononuclear cells. CD45 mRNA expression was, as expected, similar across all insulin doses, with no statistically significant differences observed compared with control mRNA levels [control ΔCt, 26.81 ± 0.78 (mean ± SE); 1 nM insulin ΔCt, 26.83 ± 0.88; 100 nM insulin ΔCt, 27.72 ± 0.69]. CD45 mRNA expression was also not different in the samples with RSG alone (10 nM RSG ΔCt, 27.2 ± 0.92) or in combination with insulin (100 nM insulin plus 10 nM RSG ΔCt, 27.53 ± 0.52).

Regulation of resistin secretion in sc adipocytes and effects of RSG

In contrast to the lack of effect on resistin mRNA expression, insulin stimulated resistin secretion in adipocytes in a concentration-dependent manner, with maximal stimulation observed at 1 μM insulin [control, 1215 ± 87 pg/ml (mean ± SEM); 1 nM insulin, 1414.0 ± 89 pg/ml; 10 nM insulin, 1478 ± 29 pg/ml (significant increase, $P < 0.01$); 100 nM insulin, 1545 ± 109 pg/ml (significant increase, $P < 0.01$); 1 μM insulin, 1797 ± 107 pg/ml (significant increase, $P < 0.001$); Fig. 3]. The time-course study (4–24 h) indicated that resistin secretion was only significantly stimulated by insulin at 24 h with 100 nM insulin (Table 2). RSG alone did not influence resistin secretion at any concentration up to 10 μM (10 μM RSG, 1139 ± 93 pg/ml; 10 nM RSG, 1195 ± 213 pg/ml). RSG (10 nM) significantly reduced the insulin-mediated increase in resistin production across the entire range of concentrations studied ($P < 0.001$; Fig. 4).

Western analysis of RELMs

Western blotting determined that both RELMα and RELMβ were recognized by the antibodies in their respective positive control lanes (10 μg protein/well; Fig. 5, I and II). However, neither RELMα nor RELMβ was expressed in adipose tissue or isolated adipose cells (10–60 μg protein/well).

TABLE 1. Resistin mRNA expression at 48 h in isolated sc adipocytes

Treatments	Resistin mRNA expression at 48 h (ΔCt mean ± SE)
Control	20.69 ± 0.31
Ins (1 nM)	20.41 ± 0.42
Ins (10 nM)	21.03 ± 0.32
Ins (100 nM)	20.49 ± 0.31
Ins (500 nM)	20.36 ± 0.48
Ins (1000 nM)	20.69 ± 0.34
Ins (1 nM) + RSG (10 nM)	21.14 ± 0.56
Ins (10 nM) + RSG (10 nM)	21.51 ± 0.47
Ins (100 nM) + RSG (10 nM)	20.53 ± 0.36
Ins (500 nM) + RSG (10 nM)	21.37 ± 0.67
Ins (1 μM) + RSG (10 nM)	20.22 ± 0.58
RSG (10 nM)	20.43 ± 0.55

TABLE 2. Resistin protein secretion over time

Time (h)	Treatment regimens (pg/ml)		
	Control	1 nM insulin	100 nM insulin
4	360 ± 50	390 ± 50	440 ± 40
12	510 ± 20	450 ± 100	510 ± 200
24	630 ± 11	720 ± 140	900 ± 100[a]

[a] $P < 0.05$.

Chronic effect of recombinant resistin on glucose uptake in differentiated preadipocytes

Chronic treatment of recombinant resistin in differentiated sc preadipocytes reduced glucose uptake and incorporation into lipid in a concentration-dependent manner (control, 17,897 ± 735 dpm; 30 ng/ml recombinant resistin, 14,984 ± 782 dpm; $P < 0.05$; Fig. 6). At lower concentrations (0.1–1 ng/ml) of recombinant resistin, no effect on lipogenesis rate was observed. Resistin had no significant effect on $^{14}CO_2$ released from ^{14}C-labeled glucose. The acute effect (14 h) of recombinant resistin in differentiated sc preadipocytes was also examined. Recombinant resistin treatment reduced glucose uptake in the differentiated sc preadipocytes (controls, 17,897 ± 735 dpm; 30 ng/ml recombinant resistin, 14,001 ± 1,006 dpm; $P = 0.0261$).

Chronic effect of recombinant resistin on lipolysis during differentiation

Assessment of glycerol release on d 12 of the differentiation studies revealed that resistin had no effect on lipolysis compared with controls (Table 3). Chronic treatment with recombinant resistin over time (12 d) did not alter lipolysis. Acute exposure to resistin also had no effect on lipolysis (data not shown).

Assessment of leptin secretion during differentiation

Previous rodent studies indicate that recombinant resistin inhibits differentiation (4); therefore, this may limit obesity at the expense of diabetes. To study this phenomenon in human cells, leptin was assessed as a time-dependent marker of differentiation in the abdominal sc preadipocytes. These studies determined that the differentiating sc preadipocytes increased leptin secretion progressively over the 12-d time course. Resistin had no effect on leptin secretion (Table 4).

Assessment of lipid accumulation during differentiation

Lipid accumulation was assessed as another marker of differentiation. Differentiating preadipocytes (in the presence or absence of recombinant resistin) accumulated lipid over time. No difference was observed in the accumulation of lipid by differentiating preadipocytes with varying concentrations of recombinant resistin (Fig. 7).

Discussion

In the present study we validated a commercially available ELISA kit for human resistin and examined circulating levels of resistin in nondiabetic and type 2 diabetic subjects. For the measurement of human serum, it was also important to establish whether there was any cross-reactivity with other

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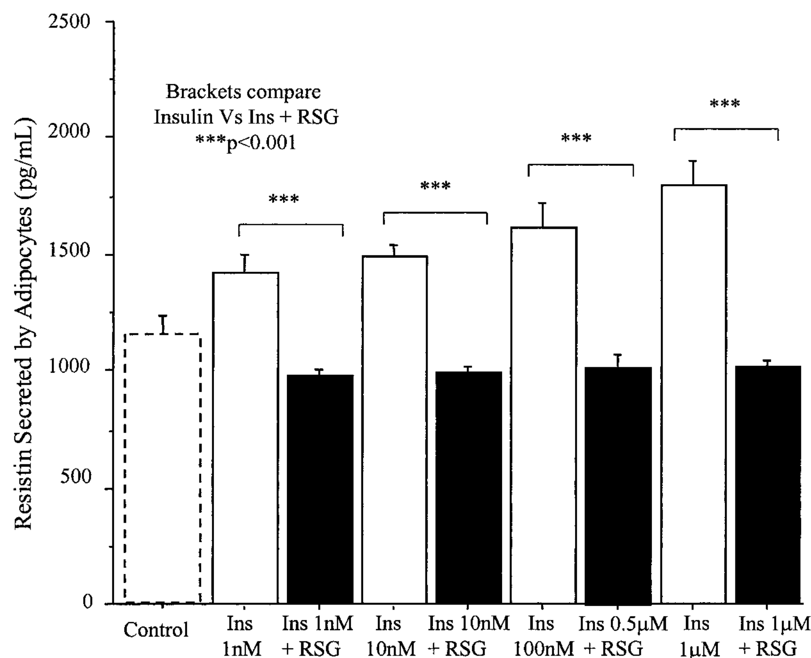

FIG. 4. This graph shows the protein secretion (±SEM) of resistin in response to insulin (1–1 μM) and insulin (1–1 μM) in combination with RSG (10 nM) in abdominal sc adipocytes. The control was abdominal sc adipocytes maintained without treatment. Values obtained with insulin alone were compared with those with insulin in the presence of RSG (***, $P < 0.001$).

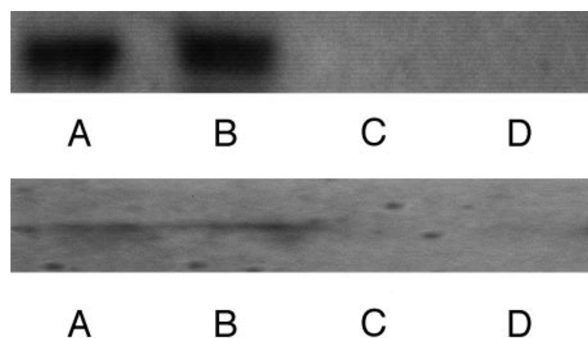

FIG. 5. Representative Western blots showing the expression of RELMα/FIZZ1 (I) and RELMβ/FIZZ2 (II) in human primary mucosal colon tissue (A) and a human lung carcinoma cell line (B). The absence of expression was noted in abdominal sc (C) and omental (D) adipose tissue. Isolated adipose cells failed to show positive protein expression (n = 4).

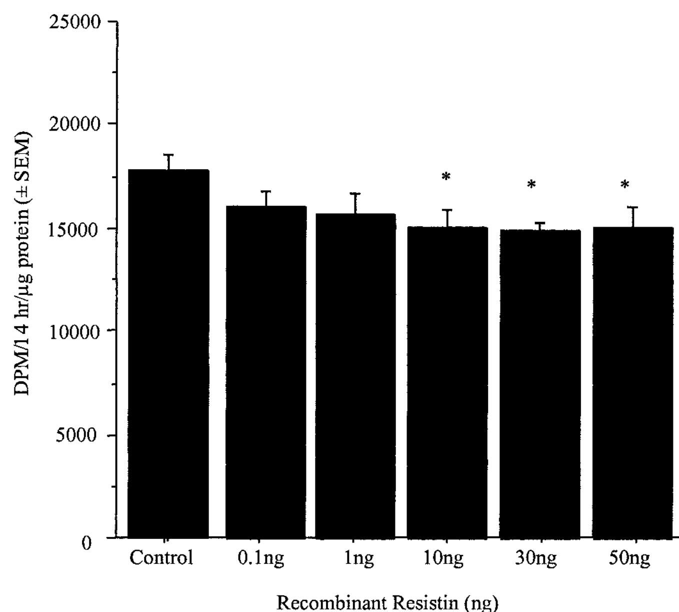

FIG. 6. Glucose incorporation into differentiated preadipocytes was measured as disintegrations per minute per 14 h per microgram of protein (±SEM). Cells were treated with varying concentrations of recombinant resistin. The control was differentiated cells maintained in the absence of resistin. Comparisons of different treatments with controls was performed using ANOVA (*, $P < 0.05$; n = 7).

RELMs in the resistin ELISA. Therefore, interference of RELMα/FIZZ 1 and RELMβ/FIZZ 2 partial peptides in the resistin assay were determined. Our studies confirmed that RELMα/FIZZ 1 and RELMβ/FIZZ 2 partial peptides at varying concentrations in either aqueous solution or serum did not interfere significantly in the resistin assay. Similarly, recombinant resistin was used to assess recovery in the ELISA, and an overall 90–98% recovery was measured.

Serum resistin was about 20% higher in type 2 diabetic subjects compared with nondiabetic subjects. However, in contrast to the strong correlation shown previously between BMI and leptin, also noted in this study, such a relationship was not observed with serum resistin (27, 28). These data suggest that in human diabetes factors other than obesity and serum insulin are likely to be the major determinants of serum resistin. In contrast, recent studies of human resistin protein expression have suggested that both adiposity and insulin are important determinants of increased resistin levels in lean and obese subjects (29). Furthermore, recent molecular genetic analysis for single nucleotide polymorphisms in the resistin gene implicate both BMI and insulin sensitivity in nondiabetic subjects, but not type 2 diabetic subjects (30–32).

Additional studies using stepwise multiple regression analysis with resistin as the dependent value, examined all biochemical and anthropometric factors as independent variables (including fasting glucose, leptin, insulin levels, and diabetic status), determined that C-reactive protein was the

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TABLE 3. The chronic effect of recombinant resistin on glycerol release in differentiated adipocytes

Treatments	Glycerol release d 12 (μmol/well · 14 h)
Control	68.8 ± 2.5
Resistin (0.1 ng)	74.6 ± 3.7
Resistin (1.0 ng)	72.7 ± 3.7
Resistin (10 ng)	71.5 ± 3.5
Resistin (30 ng)	65.1 ± 4.8
Resistin (50 ng)	64.8 ± 5.5

TABLE 4. Time-dependent increase in leptin protein secretion

Treatment regimens (resistin, ng/ml)	Leptin secretion over days (pg/ml)		
	4	8	12
Control	N/D	166 ± 32	431 ± 42
0.1	N/D	163 ± 27	385 ± 22
1.0	N/D	153 ± 35	441 ± 35
10.0	N/D	172 ± 21	465 ± 24
30.0	N/D	156 ± 24	445 ± 37
50.0	N/D	168 ± 32	448 ± 39

only significant predictor of resistin within this cohort. These data therefore indicate that in human subjects, resistin is associated more closely with inflammation.

Although no human studies have been reported to date, the role of resistin as a factor secreted as part of an inflammatory response has been examined in two recent mouse studies. These studies have addressed the effect of lipopolysaccharide (LPS), a compound of the cell wall of Gram-negative bacteria, and demonstrated that it induces an inflammatory reaction that may alter resistin expression. These studies, however, yielded conflicting results, with Rajala *et al.* (33) finding that LPS had no effect on resistin expression, whereas Lu *et al.* (34) suggested that resistin mRNA expression rose in response to LPS. The apparent difference may relate to several factors, including dose regimen and the animal model used. It should also be noted that the time point at which resistin mRNA expression was evaluated differed between the two studies, which may have influenced the findings. Lu and co-workers (34) measured resistin mRNA expression at an early time (1–8 h) and noted an increase, whereas Rajala *et al.* (33) examined the effect of LPS on resistin mRNA expression after 24 h. As LPS induces acute phase reactants over the first few hours, such a rise in resistin mRNA expression may be lost by 24 h postinjection. Further studies are required to examine this in more detail, as the present study certainly suggests a potential role for resistin as a proinflammatory factor, at least in humans.

We also examined the direct effect of insulin on adipose tissue to extend our previous findings of resistin gene and protein expression in human adipose tissue (11, 12). In the present study we determined the time course of resistin expression and secretion in cultured adipocytes and then assessed the effect of insulin and RSG on resistin expression in these human adipose-derived cells. Our data demonstrate that the expression of both protein and message were closely related over short periods of time (up to 12 h), but at 24 h, protein secretion increased while resistin mRNA remained unchanged. These results imply that resistin protein synthesis, and hence circulating concentrations, may be modified by posttranscriptional as well as transcriptional mechanisms. This apparent lack of correlation between resistin mRNA and protein in adipocytes has also been observed in mouse model studies (2, 34).

It is well established that TZDs, including RSG, function as potent insulin-sensitizing agents *in vivo* by increasing insulin-dependent glucose uptake and reducing hepatic glucose output (26, 35–37). TZD has also been shown to reduce resistin expression in 3T3-L1 adipocytes and adipose tissue *in vivo* (2). Our data indicate that, as previous rodent model data suggest, resistin secretion from sc abdominal adipocytes increases under hyperinsulinemic conditions, and that this rise is mitigated by TZDs such as RSG. The regulation of resistin expression by insulin and RSG also suggests that resistin may have a paracrine or autocrine effect in adipose tissue, a possibility that requires further study. The mechanism by which RSG suppresses insulin-stimulated, but not basal, resistin secretion from adipocytes is not clear. A recent disclosure that RSG reduced resistin expression and secretion in human macrophages *in vitro* together with the identification of a putative peroxisome proliferator-activated receptor-binding site in the promoter of the resistin gene strongly support the idea that the resistin gene can be directly transcriptionally regulated by the peroxisome proliferator-activated receptor γ-mediated mechanism (38). However, whether RSG therapy regulates circulating resistin in either nondiabetic or type 2 diabetic subjects *in vivo* remains to be determined.

In rodent models *in vivo* and in the murine 3T3-L1 adipose cell line, Lazar and co-workers (2) demonstrated that recombinant resistin reduced glucose uptake. To determine whether resistin exerts similar effects in human tissue, we examined the effects of recombinant human resistin on parameters of glucose and lipid metabolism in human adipose cells. The effects of recombinant resistin on lipogenesis and lipolysis were investigated in differentiated preadipocytes. Our findings suggest that resistin caused a modest concentration-dependent impairment of glucose metabolism after both acute and chronic exposure to the protein. Again, as with our gene and protein expression data, these findings from human cells *in vitro* are consistent with those observed in the previous mouse model studies (2). Although the effect on glucose uptake is modest, this could be relevant in poorly controlled type 2 diabetes and provide a possible additional mechanism for glucotoxicity (39). These human data may also indicate that the effects on glucose tolerance may be unrelated to the normal physiological function of resistin, and its activity may be altered to an adaptive response to starvation, as previously suggested by Lazar and co-workers for the mouse model (2).

Although resistin inhibits glucose uptake and lipogenesis by human adipocytes, there was no detectable effect on basal rates of lipolysis, as noted by Lazar (2). This may relate to the lower concentrations of recombinant resistin used in our studies. In addition, despite chronic exposure to resistin, the protein failed to influence either lipid accumulation or leptin secretion in differentiating preadipocytes, which was also apparent with assessment of leptin and resistin *vs.* BMI in our nondiabetic cohort. Additional studies examining the effect of resistin on basal and catecholamine-stimulated lipolysis in

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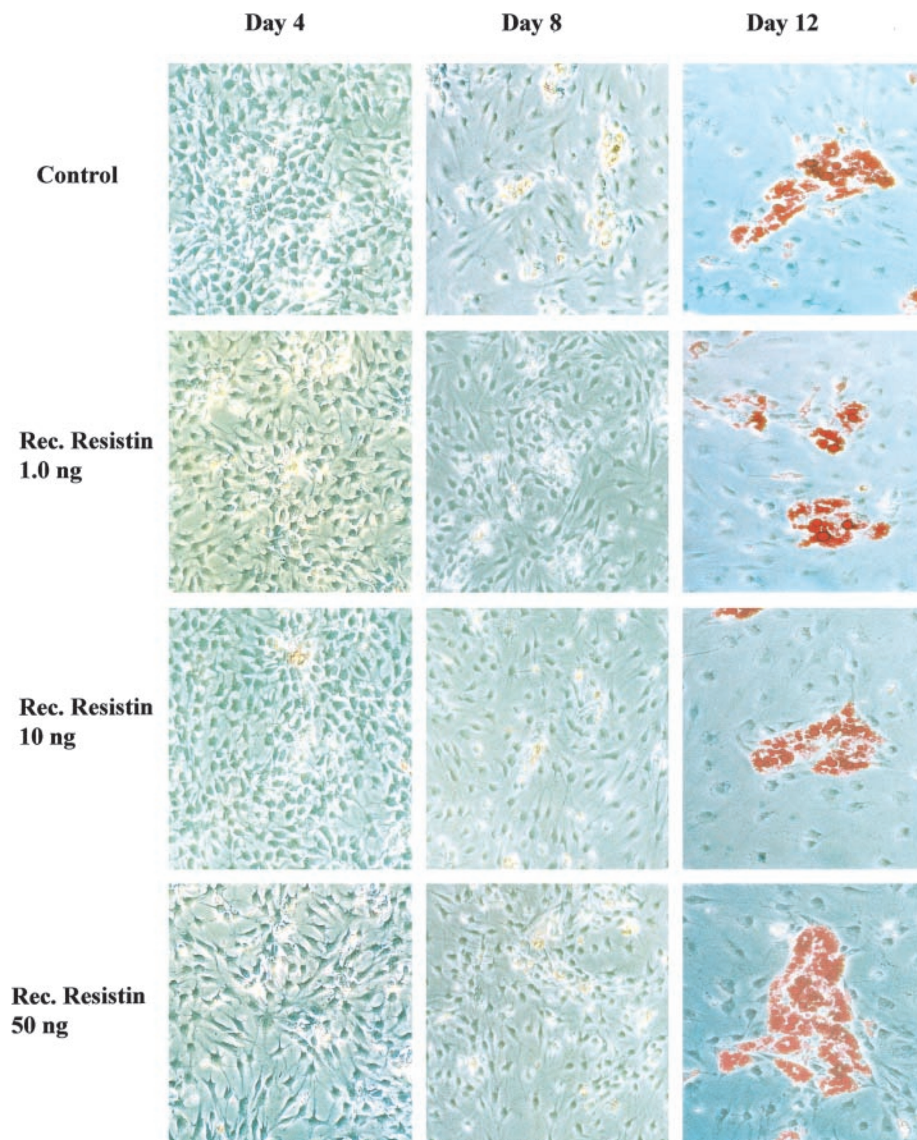

FIG. 7. Photographs show the differentiation of sc preadipocytes (Sc pa) in chemically defined differentiation medium (control) and with treatments of recombinant resistin (1, 10, and 50 ng). Day 12 differentiated Sc pa were treated with Oil Red O to define lipid accumulation (*red*). All photographs were taken using phase contrast microscopy (magnification: d 4 and 8, ×0; d12, ×20).

isolated mature adipocytes may further define the contribution of resistin to increased free fatty acids as well as glucose intolerance, as both factors are associated with insulin resistance and type 2 diabetes. The observation that resistin regulates glucose metabolism in adipocytes, albeit weakly, and at low concentrations is consistent with a receptor-mediated signaling mechanism. The nature of the resistin receptor remains unknown.

In conclusion, serum resistin is increased in type 2 diabetic subjects, in whom it is also associated with elevated C-reactive protein, suggesting that this protein may be linked to subclinical inflammation. Furthermore, resistin is actively expressed and regulated by insulin and RSG in human abdominal adipose tissue *in vitro*, with RSG suppressing the insulin-mediated stimulation of resistin expression and secretion. Similar effects have been identified with other proinflammatory cytokines, including leptin, IL-6, and TNFα (40–43), suggesting that elevated resistin serum may be related to subclinical inflammation in diabetes, a hypothesis that requires further investigation. Finally, reduction of resistin secretion may be yet another mechanism by which TZDs improve insulin sensitivity.

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