

Resistin / Fizz3 Expression in Relation to Obesity and Peroxisome Proliferator-Activated Receptor- γ Action in Humans

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Recent studies in murine models suggest that resistin (also called Fizz3 [1]), a novel cysteine-rich protein secreted by adipocytes, may represent the long-sought link between obesity and insulin resistance (2). Furthermore, peroxisome proliferator-activated receptor- γ (PPAR- γ) agonists appear to inhibit resistin expression in murine adipocytes, providing a possible explanation for the mode of action of this class of insulin sensitizers (2). Using a fluorescent real-time reverse transcriptase-polymerase chain reaction-based assay, we found that resistin mRNA levels in whole adipose tissue samples were increased in morbidly obese humans compared with lean control subjects. However, in freshly isolated human adipocytes, resistin mRNA levels were very low and showed no correlation with BMI. Resistin mRNA was undetectable in preadipocytes, endothelial cells, and vascular smooth muscle cells, but it was readily detectable in circulating mononuclear cells. Although exposure of human mononuclear cells to PPAR- γ agonists markedly upregulated fatty acid-binding protein-4 expression, these agents had no effect on mononuclear cell resistin expression. Finally, resistin mRNA was undetectable in adipocytes from a severely insulin-resistant subject with a dominant-negative mutation in PPAR- γ (3). We conclude that the recently described relationships of murine resistin/Fizz3 expression with obesity, insulin resistance, and PPAR- γ action may not readily translate to humans. Further studies of this novel class of proteins are needed to clarify their roles in human metabolism. *Diabetes* 50:2199–2202, 2001

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FABP4, fatty acid-binding protein-4; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PPAR- γ , peroxisome proliferator-activated receptor- γ ; RT, reverse transcriptase; TNF- α , tumor necrosis factor- α ; WAT, white adipose tissue.

Steppan et al. (2) recently reported a novel cysteine-rich secreted protein, which they termed resistin, the expression of which was markedly decreased by treatment of a murine adipocyte cell line with an agonist of the nuclear hormone receptor peroxisome proliferator-activated receptor- γ (PPAR- γ). Serum levels of resistin were elevated in obese mice, and immunoneutralization of circulating resistin in these animals improved insulin sensitivity. Administration of recombinant resistin impaired insulin action in vivo in mice and ex vivo in an adipocyte cell line. These observations led the authors to conclude that resistin might represent an adipocyte-derived mediator of the link between obesity and insulin resistance. They also suggested that the suppression of resistin expression by PPAR- γ agonists might explain the beneficial effects of these compounds in insulin-resistant states. Contrasting conclusions were reached by Way et al. (4), who found reduced resistin mRNA levels in white adipose tissue (WAT) of several obese rodent models. Furthermore, treating these animals with PPAR- γ agonists increased resistin mRNA levels in WAT. These discrepant observations are difficult to reconcile and indicate the need for further studies. We developed a real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)-based assay for human resistin using primers based in exons 1 and 2 of the human gene and used it to examine the expression of resistin mRNA in human tissue.

RESEARCH DESIGN AND METHODS

Adipose tissue biopsies were obtained by needle liposuction or from subjects undergoing bariatric surgical procedures (5). Whole adipose tissue samples were taken from six lean subjects (four men and two women, BMI 22–26 kg/m², four Caucasian and two African-American) and six morbidly obese subjects (five women and one man, BMI 43–63 kg/m², all Caucasian). Adipocytes and preadipocytes were isolated and cultured as previously described (6). Adipocyte samples were obtained from 14 subjects (10 women and 4 men, BMI 24–59 kg/m², all Caucasian). Adipocytes used for cell culture were isolated from five morbidly obese subjects (four women and one man, BMI 44–60 kg/m², all Caucasian). Pooled human umbilical vein endothelial cells (Clonetics) and primary arterial vascular smooth muscle cells ($n = 2$ men) were cultured according to standard protocols. Local ethical approval and informed patient consent was obtained.

Mononuclear cell isolation and culture. Peripheral blood was obtained from eight healthy subjects (four men and four women, BMI <30 kg/m², all

Caucasian). Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll gradient centrifugation. The PBMCs were transferred to 10 ml phosphate-buffered saline (PBS) and centrifuged for 10 min at 1200 rpm. The supernatant was removed, and the dilution and centrifugation were repeated three times. PBMCs were then counted and resuspended in RPMI-1640 (Sigma) with 1% charcoal-stripped fetal bovine serum (FBS), and 3×10^6 cells/well were transferred to 6-well plates. After 1 h at 37°C in a 5% CO₂ humidified incubator, nonadherent cells were removed, and the monocytes were washed twice with PBS before resuspension in RPMI-1640 with 1% charcoal-stripped FBS. Rosiglitazone, a nonthiazolidinedione PPAR- γ agonist (GL262570; a gift from T.M. Willson) (7), or vehicle was then added to each well at the desired concentrations, and RNA was isolated 24 h later.

RNA isolation. Total RNA was isolated using Qiagen reagents (Qiagen, West Sussex, U.K.). RNA samples were quantified by spectrophotometry, and integrity was assessed by agarose gel electrophoresis and ethidium bromide staining.

Quantitation of resistin and fatty acid-binding protein-4 mRNA expression by real-time quantitative RT-PCR. Total RNA (100 ng) was reverse-transcribed for 1 h at 37°C in a 20- μ l reaction containing 1 \times RT buffer (50 mmol/l Tris-HCl, 75 mmol/l KCl, 3 mmol/l MgCl₂, and 10 mmol/l dithiothreitol), 150 ng random hexamers, 1.25 mmol/l dNTP, and 200 units M-MLV RT (Promega). Reactions in which RNA was omitted served as negative controls. A reaction containing 500 ng total RNA was also included as a standard. After first-strand cDNA synthesis, this standard was serially diluted 1:2 in DNase-free water to generate a standard curve for the PCR analysis.

Oligonucleotide primers and a Taqman probe for human resistin (Fizz3) and fatty acid-binding protein-4 (FABP4) were designed using Primer Express, Version 1.0 (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences were as follows for resistin: forward 5'ATC AAT GAG AGG ATC CAG GAG 3', reverse 5'TCC AGG CCA ATG CTG CTT A 3', and probe 5'CGC CGG CTC CCT AAT ATT TAG GGC A 3'. The sequences for FABP4 were: forward 5'GGA AAA TCA ACC ACC ATA AAG AGA A 3', reverse 5' GGA AGT GAC GCC TTT CAT GAC 3', and probe 5' ACG AGA GGA TGA TAA ACT GGT GGT GGA ATG 3'. The probes were labeled at the 5' end with the reporter dye 6-carboxy-fluorescein (FAM) and at the 3' end with the quencher 6-carboxy-tetramethyl-rhodamine (TAMRA). Oligonucleotide primers and a Taqman probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Perkin-Elmer.

PCR was carried out in duplicate for each sample on an ABI 7700 sequence detection system (Perkin-Elmer), and all reactions were performed on at least two occasions. Each 25- μ l reaction contained 4 μ l first-strand cDNA, 1 \times PCR master mix, 300 nmol/l of each forward and reverse primer, and 150 nmol/l Taqman probe. All reactions were carried out using the following cycling parameters: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. After PCR, standard curves were constructed from the standard reactions for resistin or FABP4 and GAPDH by plotting values for Ct (the cycle number at which the fluorescence signal exceeds background) versus log cDNA input (in nanograms). The Ct readings for each of the unknown samples were then used to calculate the amount of resistin, FABP4, or GAPDH relative to the standard. For each sample, results were normalized by dividing the amount of resistin or FABP4 by the amount of GAPDH. Resistin mRNA was only considered to be detectable in samples in which the duplicate Ct values did not vary by >5%. At Ct values near 40 (i.e., 35+ PCR cycles), the duplicates tend to show considerable variation and are not reproducible; we consider resistin mRNA to be undetectable in these circumstances.

Statistical analysis. The analysis of variance test was used to compare resistin mRNA expression in subcutaneous whole adipose tissue from lean and obese subjects.

RESULTS

Human resistin mRNA was undetectable in adipose tissue biopsies from lean subjects ($n = 6$). In contrast, resistin mRNA was consistently detectable in adipose tissue obtained at surgery from morbidly obese subjects ($n = 6$) (Fig. 1A). Levels of resistin mRNA in omental and subcutaneous tissue from individual subjects were tightly correlated (Fig. 1B). Although this relationship with obesity is consistent with the murine data (2), we could find no relationship between adiposity and resistin mRNA expression in freshly isolated adipocytes obtained from an independent group of 14 subjects ranging in BMI from 22 to 59 kg/m² (Fig. 2). In fact, resistin mRNA was only detected in

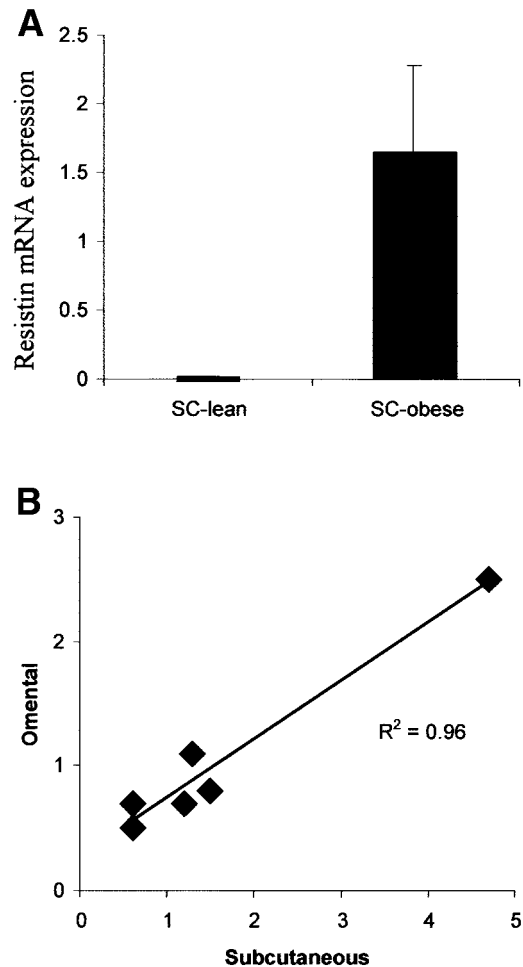


FIG. 1. Resistin mRNA expression in whole adipose tissue. Resistin mRNA levels were measured by real-time RT-PCR and expressed relative to levels of GAPDH mRNA (arbitrary units). **A:** Resistin mRNA levels (means \pm SE) in subcutaneous (SC) whole adipose tissue samples from six lean (BMI 22–26 kg/m²) subjects compared with subcutaneous samples from six morbidly obese (43–63 kg/m²) subjects ($P < 0.01$). **B:** Correlation between resistin mRNA levels in subcutaneous and omental subjects whole adipose tissue from six morbidly obese (43–63 kg/m²) subjects.

isolated adipocytes from four subjects. The observed increase in resistin expression in WAT from morbidly obese individuals may relate to resistin expression in other cell types in adipose tissue. Indeed, resistin mRNA levels were higher in the stromovascular fraction of WAT biopsies than in the isolated adipocytes (data not shown). This is in direct contrast to what was reported in rodent adipose tissue (8). We went on to examine a range of human cell types representing the constituents of adipose tissue for resistin expression and found that it was undetectable in preadipocytes, in vitro differentiated preadipocytes, and vascular endothelial and vascular smooth muscle cells. In contrast, resistin mRNA was relatively abundant in PMBCs (eight of eight subjects tested). The extremely low levels of resistin expression in adipocytes in addition to interindividual variability made it difficult to compare adipocyte and monocyte resistin mRNA levels directly. However, our finding of predominant expression of human resistin in mononuclear cells and low/absent expression in human adipocytes was supported by a bioinformatic search for resistin sequences undertaken in

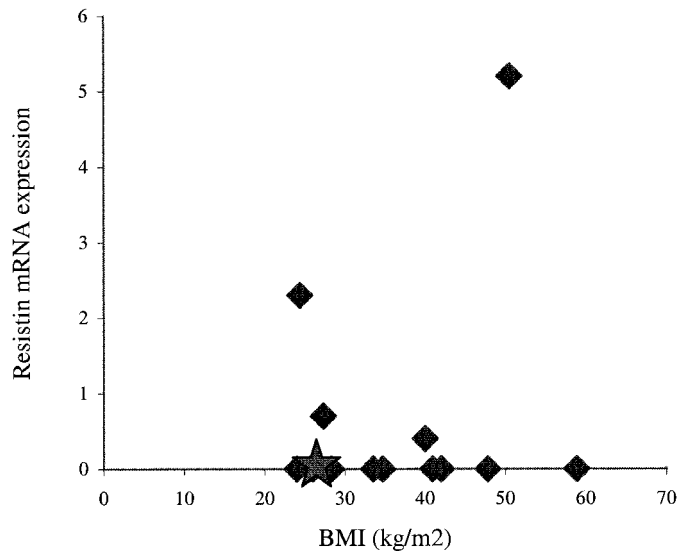


FIG. 2. Effect of increasing adiposity on adipocyte resistin mRNA expression. Resistin mRNA levels were measured by real-time RT-PCR and expressed relative to levels of GAPDH mRNA (arbitrary units). Resistin mRNA levels in isolated subcutaneous adipocytes from 14 subjects (BMI 24–59 kg/m²). *, adipocytes from a subject with a dominant-negative PPAR- γ mutation (P467L) (3).

>1,375 human cDNA libraries. cDNAs encoding resistin were found in only 10 of these libraries, 4 of which were of lymphoid/monocyte origin. Resistin sequences were not found in any of the 17 adipocyte-derived cDNA libraries (I. Barroso, Incyte Genomics; personal communication).

PPAR- γ agonists were initially reported to suppress resistin expression in murine adipocytes (2), although a second study suggested that PPAR- γ agonists increase adipose tissue resistin mRNA levels (4). The latter observation may simply reflect increased adipocyte differentiation, which was associated with increased resistin expression in two reports in rodent models (2,8). Because resistin was undetectable in cultured human adipocytes (48 h, $n = 5$ morbidly obese individuals), we examined the effects of PPAR- γ agonists in human mononuclear cells. Whereas expression of FABP4 (human aP2), a known

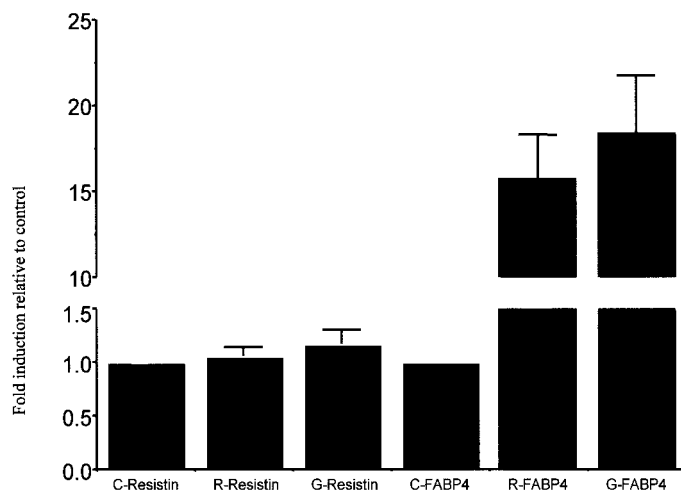


FIG. 3. Effect of PPAR- γ agonists on resistin and FABP4 mRNA levels in mononuclear cells. Human monocytes were exposed to 100 nmol/l rosiglitazone (R), 100 nmol/l G1262570 (G), or vehicle (control [C]) for 24 h. Resistin and FABP4 mRNA levels (means \pm SE) were measured by real-time RT-PCR and normalized to the control samples ($n = 3$).

PPAR- γ target, was strikingly increased by 24 h of treatment with two independent PPAR- γ agonists, resistin mRNA levels were unchanged (Fig. 3). Basal levels of resistin mRNA are 10- to 15-fold higher than FABP4 in cultured human monocytes, whereas these two mRNAs are expressed at similar levels in PPAR- γ agonist-treated cells (data not shown). Finally, we studied a subject with severe insulin resistance resulting from a dominant-negative mutation in PPAR- γ (3). The causal role of the PPAR- γ mutation in the insulin resistance of the proband is supported by its cosegregation with severe insulin resistance in family members as young as 3 and 7 years of age (data not shown). Resistin mRNA was undetectable (Fig. 2) in subcutaneous adipocytes obtained from the proband.

DISCUSSION

In summary, our data suggest the following conclusions. First, although resistin expression in adipose tissue from morbidly obese subjects is increased in comparison to lean subjects, there is no correlation between BMI and resistin expression in adipocytes per se. One possible explanation for this observation is that adipose tissue in morbidly obese individuals houses a greater proportion of mononuclear cells (monocytes, macrophages, and lymphocytes). This hypothesis is supported by the fact that human resistin mRNA was more readily detectable in the stromovascular fraction of WAT biopsies. Another possibility is that the process of adipocyte isolation alters resistin mRNA levels. Second, resistin mRNA is more abundant in circulating mononuclear cells than in adipocytes, a finding supported by a bioinformatic search of cDNA libraries. Third, both pharmacological studies in human monocytes and an examination of adipocytes from a patient with a genetic defect in PPAR- γ failed to provide any support for the notion that human resistin expression is directly altered by PPAR- γ action, at least in these cell types. Finally, resistin expression in subcutaneous and omental adipose tissue from morbidly obese subjects is very similar, suggesting that resistin is unlikely to explain the particular link between visceral adiposity and insulin resistance. Before drawing any firm conclusions from our data, we should emphasize that all our data are restricted to resistin mRNA levels, and it is possible that direct measurements of resistin protein will reveal different relationships. Having said that, adipocyte-secreted proteins usually show a strong correlation between mRNA and protein levels (9–12).

The published sequence of murine resistin is identical to a member (Fizz3) of the recently reported Fizz family of secreted cysteine-rich proteins (1), the index protein of which (Fizz1) was isolated from inflamed lung. Our findings suggest that the strong relationship among obesity, adipocyte resistin expression, systemic insulin resistance, and the amelioration of the latter by PPAR- γ agonists reported in the mouse (2) may not translate readily to the human situation. In fact, interspecies differences in metabolism are well recognized. For example, whereas murine models of insulin resistance show dramatic responses to PPAR- γ agonists, such agents have more modest effects in humans (13). The findings with resistin are strikingly reminiscent of tumor necrosis factor- α (TNF- α), another cytokine expressed in monocytes and adipose tissue (10).

Although interference with adipocyte TNF- α action strikingly ameliorates the insulin resistance of murine obesity (14), TNF- α is expressed at much lower levels in human adipose tissue (15), and inhibition of TNF- α has a negligible effect on the insulin resistance of obese humans (16). Although uncertainties persist regarding the broader role of this protein in normal and disordered metabolism, we suggest that the use of the term "resistin" for the human protein may be somewhat premature. The term "Fizz3" has the advantage of identifying this protein as one member of a novel human cytokine family, the biological roles of which remain to be fully elucidated.

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