

A Promoter Genotype and Oxidative Stress Potentially Link Resistin to Human Insulin Resistance

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Insulin resistance is a component of type 2 diabetes and often precedes pancreatic β -cell failure. Contributing factors include obesity and a central pattern of fat accumulation with a strong genetic component. The adipocyte secreted hormone resistin has been proposed as a link between the adipocyte and insulin resistance by inhibition of insulin-stimulated glucose uptake and/or blocking adipocyte differentiation. Here we report that the G/G genotype of a single nucleotide polymorphism (SNP) in the promoter of the human resistin gene, $-180C>G$, had significantly increased basal promoter activity in adipocytes. These data were recapitulated in vivo, where G/G homozygotes had significantly higher resistin mRNA levels in human abdominal subcutaneous fat. A significant interaction was also found between the $-180C>G$ SNP, a marker of oxidative stress (NAD[P]H quinone oxidoreductase mRNA) and homeostasis model assessment of insulin resistance. In addition, resistin mRNA was positively and independently correlated with insulin resistance and hepatic fat as measured by liver X-ray attenuation. These data implicate resistin in the pathophysiology of the human insulin resistance syndrome, an effect mediated by the $-180C>G$ promoter SNP and potentially cellular oxidative stress. *Diabetes* 52:1611–1618, 2003

The hormone resistin is a member of a novel family of cysteine-rich secreted proteins associated with pulmonary inflammation (FIZZ3) and expressed in the murine small bowel and adipose tissue (1). Resistin was downregulated in the mouse by thiazolidinediones (TZDs), which are agonists for the antidiabetic peroxisome proliferator-activated receptor γ (PPAR- γ), and was proposed to link obesity to diabetes (2). The latter findings were contradicted in rodent models of obesity in which PPAR- γ agonists augmented expression of resistin (3). At the genic level, single nucleotide polymorphisms (SNPs) in noncoding regions of the human resistin gene were either not significantly associated with insulin resistance (4,5) or associated with an insulin sensitivity index in the case of a different promoter SNP

from the one that we present in this article (6). Resistin expression in humans has been reported at low levels in the adipose tissue of some but not all humans (7,8), and its reduced expression has also been proposed as a hallmark of obesity (9). In another study, resistin mRNA was not related to insulin resistance when using RNA isolated from cultured adipocytes (10) but was upregulated by acute hyperglycemia in various mouse adipose depots (11). Using 32 adipose tissues and quantitative PCR, however, there was increased amount of resistin mRNA in abdominal depots compared with thigh depots, suggesting an increased risk for type 2 diabetes as a result of central obesity and higher resistin (12). The genomic organization and regulation of the murine and human resistin genes are divergent and may explain these discrepant findings (13).

Resistin was also reported as a cysteine-rich adipose tissue-specific secretory factor that blocks adipocyte differentiation (14). This finding provides an appealing functional role for resistin by which ectopic fat accumulation occurs as a result of impaired lipid storage in adipocytes. Indeed, failure of adipocyte differentiation has been proposed as a cause of type 2 diabetes (15), possibly through an ectopic overload of fatty acids and lipotoxicity of nonadipose tissues (16). A role for resistin could therefore be envisioned in the prediabetic syndrome of insulin resistance 1) by virtue of its ability to block adipocyte differentiation or 2) as a direct result of increased circulating levels and insulin resistance.

Cellular oxidative stress is the result of normal cellular processes and occurs at the level of the mitochondria when proton potential is high (17), when free radicals are generated at the plasma membrane (18), or when cells are exposed to environmental toxins (19). Cellular responses to limit oxidative stress are grouped into phase I and phase II enzymes (20), the latter including heme oxygenase and NAD(P)H:quinone reductase (EC 1.6.99.2). NAD(P)H:quinone oxidoreductase 1 (NQO-1), a 274-amino acid flavoprotein, is a prototypical phase II enzyme that is induced by electrophiles such as tert-butyl hydroxyquinone and oxygen free radicals (21). NQO utilizes NAD(P)H as an electron donor and acts to reduce and detoxify quinones and their derivatives. NQO-1 knockout mice exhibit alterations in the intracellular redox state with a resulting phenotype of reduced visceral adipose tissue mass (22). Combined with a growing body of evidence linking insulin resistance and oxidative stress (23), there may be a prominent role for free radicals in the regulation of adipocyte function. Oxidative stress impairs insulin action in adipocytes in vivo (24–26), and in vivo, treatment with the antioxidant lipoic acid improves insulin-stimulated glucose disposal (27). Given the putative role of resistin in

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CT, computed tomography; DEXA, dual X-ray absorptiometry; HOMA-IR, homeostasis model assessment of insulin resistance; NQO, NAD(P)H:quinone oxidoreductase; PPAR, peroxisome proliferator-activated receptor; SNP, single nucleotide polymorphism; TZD, thiazolidinedione.

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insulin resistance, oxidative stress might also have an impact on endogenous expression of resistin in the adipocyte.

To understand better the role of resistin in human insulin resistance, we examined the promoter of resistin for the presence of functional mutations. We report the identification of an SNP in the proximal promoter of the human resistin gene. The possible association of this SNP with obesity phenotypes was evaluated in a cohort of 978 individuals. Analysis of the homozygous genotypes in cell models showed that this is a functional SNP that affects promoter activity and could therefore affect expression of the gene in vivo. This hypothesis was tested in a second cohort of 58 individuals in whom resistin mRNA was measured in subcutaneous fat and related to the -180 SNP. Given 1) the potential link between oxidative stress and insulin resistance and 2) a lack of interaction between resistin expression in human adipocytes in vitro and known regulators of adipocyte function (glucocorticoids, PPAR agonists, cytokines, etc.; data not shown), we next tested the hypotheses that there might be significant interactions among the -180 SNP, resistin mRNA, and oxidative stress in vivo using NQO mRNA as a measure of oxidative stress. The results presented here provide insights into a potential role for resistin in the pathophysiology of the human insulin resistance syndrome.

RESEARCH DESIGN AND METHODS

Promoter of resistin. The gene structure and promoter region for resistin/FIZZ3 was determined according to sequences in the Bacterial Artificial Chromosome (BAC) with GenBank accession number AC008763, sequences for the C/EBP Σ -regulated myeloid-specific secreted cysteine-rich protein precursor gene (HXCP1) with accession number AF352730, and BLAST analyses of the cDNA with accession number NM_020415. Nucleotide +3,168 on sequence with accession number AF352730 was designated as the putative transcription start site (nucleotide number, “-1”).

Sequence alignments and algorithmic analyses. Sequences alignments between the mouse and human resistin promoter orthologous regions were performed with a web-based algorithm (saturn.med.nyu.edu/searching/prsa.cgi; Skirball Institute of Biomolecular Medicine, NY University School of Medicine, New York, NY). The human DNA sequences used were from the GenBank accession number AF352730, and the mouse sequence was extracted from the publication by Hartman et al. (28). Algorithmic analyses to identify predicted recognition binding sites for transcription factors in the promoter of resistin were performed with the TESS software (29).

The $-180C>G$ promoter polymorphism. The $-180C>G$ SNP in the human resistin promoter was identified in the SNP database (Sanger Center, Cambridge, U.K.) with dbSNP accession number 273660. The positional assignment for this SNP was based on the assignment of the transcription start site at nucleotide 3,168 in the sequence with accession number AF352730.

Genotyping of individuals for the $-180C>G$ SNP was performed on a LI-COR DNA Analyzer 4200 (Lincoln, NE). Amplicons were generated with the primers FZ3P1:5'-TTTTGTTCATGTTTGCATCAGC-3' and FZ3P2:5'-GGGCTCAGCTAACCAAAT-3'. PCR conditions were as follows: one cycle at 95°C for 4 min, followed by 30 cycles, each consisting of denaturation step at 95°C for 1 min, an annealing step at 60°C for 1 min, and an extension step at 72°C for 1 min. The sequencing reactions were performed as prescribed by the manufacturer (LI-COR, Lincoln, NE) with the following primer tagged with IRDye 800 at 795 nm: 5'-gaccagtctctggacatgaa-3'.

Transfection reporter constructs. Reporter constructs were generated by amplification of genomic DNA from two homozygous individuals (C/C and G/G) and cloning of the PCR amplicons into the pGL3-basic luciferase reporter vector (Promega, Madison, WI). The PCR primers were the FZ3P1 and FZ3P2 primers described above with the addition of leader sequences that corresponded to the *SacI* and *XhoI* restriction enzymes to facilitate directional cloning into the pGL3-basic vector. PCR products and a pGL3-basic vector plasmid preparation were treated with the two enzymes as prescribed by the manufacturer (New England Biolabs, Beverly, MA), purified from agarose gel slices, and incubated into a ligation reaction at 16°C overnight in the presence of T4 DNA ligase (New England Biolabs). After transformation of competent DH5 α cells (GIBCO-BRL, Gaithersburg, MD), transformants were grown in

LB/Amp media and preparations were made for the plasmids representing the two genotypes: $-303/+27(C/C)$ and $-303/+27(G/G)$. The genotypes of the two constructs were confirmed by bidirectional sequencing of the plasmids (Perkin Elmer, Wellesley, MA).

Cell culture and luciferase assay 3T3-L1 cells were differentiated by incubation for 48 h in the following reagents: DMEM (Cellgro; Fisher, Pittsburgh, PA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, 830 nmol/l insulin, and 2 μ mol/l dexamethasone. Subsequently, the dexamethasone was removed from the media and the cells were maintained in the differentiated state in the presence of insulin for an additional 10 days. The cells were initially cultured in plastic dishes (10-cm diameter) at 37°C in a humidified atmosphere with 5% CO $_2$ in the air. For the transfections, differentiated (grown an additional 10 days after removal of dexamethasone) 3T3-L1 cells were grown to 85% confluence in six-well (34.8-mm diameter per well) plates (Corning, Corning, NY). Cells were transiently cotransfected with the constructs and β -gal plasmids for 24 h in the absence of serum using the Geneporter2 transfection reagent (Gene Therapy Systems, San Diego, CA), as previously described (30). According to the manufacturer of the kit (Gene Therapy Systems), the transfection efficiencies of the 3T3-L1 cells were estimated at 30–40% and 80–90%, respectively, using a β -gal reporter plasmid. The media were then supplemented with 20% FCS for 24 h. Cells were harvested using 1 \times Geneporter2 lysis buffer, and the lysates were assayed for luciferase and β -galactosidase activities, as prescribed by the assay manufacturer (Promega, Madison, WI) in a luminometer (ZyLux, Pforzheim, Germany). The pGL3-basic vector was used as negative control and the pGL3-promoter vector was used as a positive control.

Luciferase activity measurements were normalized to β -galactosidase values. Luciferase activity was also measured for untransfected cells. Each transfection was carried out in duplicate, and the experiments were repeated a minimum of five times for each construct, in each cell line.

The mean luciferase relative activity for each construct was calculated as a fold increase over the negative control empty vector (pGL3-basic). The data presented represent the means of five duplicate independent transfection experiments per construct.

Human studies. The Pennington Institutional Review Board approved these studies, and all participants provided written informed consent before participation. Vital signs, laboratory values, body fat, and visceral adipose tissue mass were measured as previously described (31).

Association study (cohort 1). The population consisted of 978 individuals who enrolled in studies at the Pennington Biomedical Research Center during the previous 5 years. Each participant signed informed consent. Volunteers were excluded for a diagnosis of diabetes as defined by a fasting plasma glucose >126 mg/dl. Blood glucose and insulin values were available for 671 of these individuals. Body composition was determined by dual X-ray absorptiometry (DEXA), and visceral adipose tissue (cm 2) was measured by single-slice computed tomography (CT) scanning as previously described (31).

Measurement of resistin mRNA in human adipose tissue (cohort 2). Cohort 2 included 58 healthy overweight and obese men and women. Each participant signed informed consent. Body composition was determined by DEXA, and visceral adipose tissue (kg) was measured by multislice CT scanning as previously described (31). Liver fat (X-ray attenuation value; Houndfield Units) was measured by CT scanning with splenic X-ray attenuation as an intrascan control. A low liver:spleen ratio is indicative of a fatty liver, whereas a high liver:spleen ratio is indicative of low fat in the liver (32). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as the product of fasting insulin (μ U/ml) and glucose values (mmol) divided by 22.5 (33).

After local anesthesia (according to institutional guidelines), adipose tissue was collected by needle biopsy of the superficial subcutaneous adipose tissue lateral to the umbilicus. Total RNA was isolated from ~ 50 mg of frozen tissue using Trizol (Life Technologies, Gaithersburg, MD). RNA was dissolved in Formazol and stored at -80°C . The concentration and quality of the RNA were determined spectrophotometrically. The TaqMan Real Time RT-PCR technique was used to measure quantitatively the levels of human resistin mRNA (Applied Biosystems, Foster City, CA). The primer probe set for human resistin was designed using the Primer Express software (Perkin Elmer) as follows: forward primer 5'-AGCATCAATGAGAGGATCCA-3', reverse primer 5'-TCCAGGCCAATGCTGCTTA-3', and the probe 5'-FAM-TCGCCGGCTCCC TAATATTTAGGGCA-bhq-1-3' (Biosearch Technologies, Novato, CA) that spans the exon 2/exon 3 boundary. The RT-PCR was carried out in one tube using the ABI PRISM 7700 SDS instrument (Applied Biosystems) using 10 ng of total RNA as the template. The master mix included 300 nmol/l primers, 100 nmol/l probe, reverse transcriptase MuLV, RNase inhibitor, and AmpliTaq GOLD (Applied Biosystems). The Ct value for every sample was measured in duplicate, and resistin mRNA levels were normalized to cyclophilin mRNA levels (cat. no. 4310883E; Applied Biosystems).

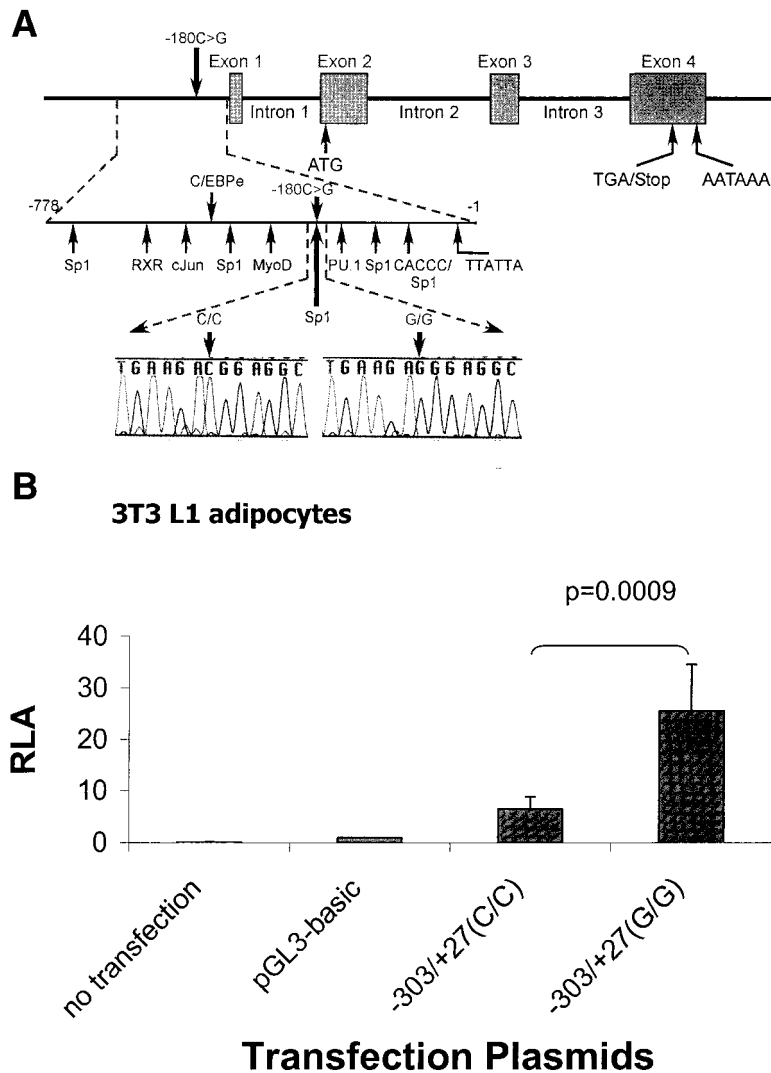


FIG. 1. Structure of the human resistin gene and functional properties of the $-180C>G$ SNP. **A:** Resistin consists of three coding exons and a 5' noncoding exon (top). The relative positions of the putative binding sites for transcription factors are indicated in the middle panel. The position of the $-180C>G$ SNP and electropherograms (bottom) of the two homozygous genotypes are shown. **B:** Luciferase activity was measured in transiently transfected 3T3-L1 cells. The data shown represent the means of a minimum of five duplicate experiments per transfection construct. RLA, relative luciferase activity, over the control pGL3-basic vector. Statistical significance between the two constructs representing the two genotypes (C/C and G/G) was calculated by the Student's *t* test.

Measurement of NQO mRNA (cohort 2). The same procedures as described above for resistin RNA were followed for the measurement of NQO mRNA using the following primers: forward primer 5'-TCATTCTCTGGCCAA TTCAGAGT-3', reverse primer 5'-GGAGTGTGCCCAATGCTATATG-3', and the probe 5'-FAM-TCTGTGGCTTCCAAGTCTTAGAACCTCAACTG-BHQ1-3' (Biosearch Technologies).

Statistical analyses. Genotype relationships with clinical and laboratory variables were determined using one-way ANOVA, two-way ANOVA, or mixed models (JMP v 4.0; SAS Institute, Cary, NC). Multivariate regression was used to test the significance of the relationship of resistin mRNA to HOMA-IR and liver fat including body fat, visceral fat, and sex in the models. Sample means are presented unless otherwise noted; however, variables were log-normalized before analysis where necessary. The results of the general linear model are graphically presented as leverage plots that illustrate the residuals of the independent variable after adjusting for the other variables in the model. Analyses were repeated for cohort 2 using ethnicity (race) and sex as covariates or using the whites only (also adjusted for sex). The data could not be analyzed for the blacks alone because of their low numbers in cohort 2 (CC = 0, GC = 11, GG = 1).

RESULTS

A polymorphism, $-180C>G$, in the promoter of resistin. The gene structure of the human resistin and the minimal promoter of the gene were determined from known genomic and cDNA sequences (Fig. 1A). Several putative binding sites for transcription factors and a noncanonical TATA-box (TTATTA) were identified by algorithmic analyses including a recognition domain for

the transcription activators of the C/EBP family (34) that is commonly found in fat cells (Fig. 1A). Putative recognition binding sites for the Sp1/Sp3 family of transcription factors were found at high frequencies (Fig. 1A).

A search of the human SNP database (dbSNP) was performed, and a polymorphism was identified 180 nt upstream of the putative transcription start site, where the cytosine was substituted by a guanine ($-180C>G$; Fig. 1A). This substitution resulted in gain/loss of recognition binding sites for several transcription factors (as predicted algorithmically), including the multifaceted specific protein, Sp1, X2 box binding protein, MaIT, R1-R2, TCF-1, TFII-I, and CAC binding protein. Alignments of the proximal promoters of the human and mouse resistin orthologs showed an overall conservation of the sequences at the 57.6% level, with higher conservation (88%) of the sequences flanking the TTATTA-box but lower conservation (50%) of the sequences flanking the $-180C>G$ SNP region. The SNP itself was not conserved between the two species.

Functional properties of the $-180C>G$ SNP. The impact of the $-180C>G$ SNP on promoter activity of the corresponding region was assessed through two identical constructs (330 nt long) in the pGL3-basic vector that

TABLE 1
Cohort 1: population characteristics according to the three $-180C>G$ genotypes

	C/C	C/G	G/G	<i>P</i>
<i>n</i> (frequency %)*	487 (49.8)	400 (40.9)	91 (9.3)	
Sex (M/F)	299/188	272/128	59/32	
Ethnicity (B/W)	79/408	74/326	18/73	
Age (years)	48.5 ± 0.48	49.41 ± 0.49	47.91 ± 1.02	NS
BMI (kg/m ²)				
M	28.9 ± 0.24	28.8 ± 0.26	29.3 ± 0.62	NS†
F	29.1 ± 0.34	28.6 ± 0.35	28.5 ± 0.82	NS
Fatness (%)				
M	29.1 ± 0.54	30.2 ± 0.66	32.3 ± 1.33	NS‡
F	44.8 ± 0.43	44.0 ± 0.45	41.9 ± 0.97	NS
VAT (cm ²)§	120.0 ± 3.14	114.5 ± 3.45	109.7 ± 6.27	NS
Glucose (mmol)	5.5 ± 0.02	5.46 ± 0.03	5.37 ± 0.06	NS
Insulin (pmol/l)	76.8 ± 2.9	85.4 ± 3.3	63.8 ± 7.1	NS

Data are means ± SE, except body fat and visceral fat, which are presented as least squares means ± SE. *P* values are by one-way ANOVA, except where noted. *Genotype frequencies are in Hardy-Weinberg equilibrium; †*P* = 0.07 by ANOVA and *P* = 0.03 between GG and CC by post hoc analysis, in white men only; ‡*P* = 0.017 by ANOVA and *P* = 0.025 between GG and CC by post hoc analysis, in white men only; §visceral abdominal tissue (VAT; measured at L4-5) was adjusted for total body fat and sex. NS, not significant.

differed only by the polymorphism $-303/+27(C/C)$ and $-303/+27(G/G)$. The luciferase reporter gene was used to measure promoter activity. Differentiated 3T3-L1 cells were transiently cotransfected with the promoter constructs and control vectors. Luciferase values were averaged, and relative luciferase activity was calculated as a fold increase over the activity of the pGL3-basic vector. The $-303/+27(G/G)$ construct had fourfold higher luciferase activity than the $-303/+27(C/C)$ construct in the 3T3-L1 adipocytes (Fig. 1B).

The $-180C>G$ SNP and human fatness. To explore the effects of the $-180C>G$ SNP on human physiology, we genotyped 978 individuals (cohort 1; Table 1) with known body composition phenotypes (percentage fat and visceral adipose tissue by CT scanning). Genotype frequencies (CC = 49.8; CG = 40.9; GG = 9.3) were in Hardy Weinberg equilibrium. The G/G genotype of the $-180C>G$ SNP was significantly associated with higher BMI and percentage body fat in white men only but not in women or in blacks of any sex (Table 1). However, after Bonferroni corrections for multiple testing were performed, this association was not significant (Table 1). No significant effects of the $-180C>G$ SNP were found on fasting insulin values, with or without adjusting for the major effects of sex, body fat, and visceral fat.

Resistin expression in human adipose tissue. A different cohort consisting of 58 unrelated individuals (cohort 2; Table 2) was used to measure resistin expression using total RNA isolated from subcutaneous fat biopsies and real-time RT-PCR. Resistin expression was highly correlated between gluteal femoral and subcutaneous abdominal depots ($R^2 = 0.59$, *P* = 0.0001; data not shown). Genotype frequencies were in Hardy Weinberg equilibrium (Table 2) but differed from those in cohort 1 probably because of the small sample size and a recruitment bias in the sample for overweight and hyperinsulinemic individuals (that tended to be G/G homozygotes; Table 2). Data were adjusted for ethnicity and sex. There was no rela-

tionship between resistin mRNA and age, body fatness, visceral adiposity, BMI, a serum marker of inflammation (C-reactive protein; data not shown), or adipose tissue TNF- α mRNA expression (data not shown). However, there was a genotype effect where G/G homozygotes had significantly higher resistin mRNA than alzygotes before (data not shown) or after adjusting the statistical model for ethnicity and sex (Table 2). When the same analysis was performed for whites only, G/G homozygotes still had significantly higher resistin expression than alzygotes (Table 2). After multiple testing was adjusted for (10 tests), the *P* value remained significant for the ethnicity-adjusted dataset (*P* = 0.02) and for the whites only (*P* = 0.02). The same analysis could not be performed for blacks because of the low numbers (Table 2).

Interactions among the $-180C>G$ SNP, oxidative stress, and resistin. There was a large degree of variability in the levels of resistin mRNA (Fig. 2A, inset), suggesting that another factor might interact with the $-180C>G$ promoter SNP to regulate resistin mRNA. Given the recent attention given to oxidative stress as an element of the insulin resistance syndrome, we hypothesized that oxidative stress might interact to increase resistin mRNA. To test this hypothesis, we measured NQO mRNA (a measure of oxidative stress) in subcutaneous fat of cohort 2 and related the genotype and NQO mRNA to resistin expression.

NQO mRNA was divided into high and low expressors (i.e., upper and lower 50th percentiles) to create a basis for comparisons. NQO mRNA was not significantly different among the three genotypes (Table 2). Using mRNA as the dependent variable, we observed significant effects of both the genotype and oxidative stress (genotype, *P* = 0.005; NQO, *P* = 0.0008; NQO \times genotype, *P* = 0.08 by ANOVA). After ethnicity and sex were adjusted for, a similar result was observed (genotype, *P* = 0.006; NQO, *P* = 0.0007; NQO \times genotype, *P* = 0.08) (Fig. 2A). However, when whites only (adjusted for sex) were tested, there was a significant interaction among genotype, NQO mRNA, and

TABLE 2
Cohort 2: population characteristics according to the three $-180C>G$ genotypes

	C/C	C/G	G/G	<i>P</i>
<i>n</i> (frequency %)*	19 (33%)	29 (50%)	10 (17%)	
Sex (F/M)	3/16	9/20	4/6	
Ethnicity (B/W)	0/19	10/19	1/9	
Age (years)	43.9 ± 2.5	41.5 ± 2.1	39.6 ± 3.4	NS
BMI (kg/m ²)	32.1 ± 0.9	32.5 ± 0.7	32.2 ± 1.8	NS
Fatness (%)	42.7 ± 1.8	39.2 ± 1.3	41.2 ± 2.6	NS
Insulin (pmol/l)	96.9 ± 13.5	95.4 ± 9.6	106.2 ± 19.8	NS
Glucose (mmol)	5.5 ± 0.2	5.6 ± 0.1	5.8 ± 0.3	NS
HOMA-IR (AU)	3.2 ± 0.6	3.3 ± 0.4	3.7 ± 0.8	NS
Liver/spleen (AU)	1.29 ± 0.1	1.20 ± 0.07	1.14 ± 0.14	NS
VAT (kg)†	3.8 ± 0.5	3.7 ± 0.3	3.9 ± 0.6	NS
NQO mRNA (AU)	0.30 ± 0.04	0.30 ± 0.03	0.36 ± 0.06	NS
Resistin mRNA (AU)	1.01 ± 1.1	1.97 ± 0.8	6.3 ± 1.4‡	0.002
Resistin mRNA (AU)§	0.75 ± 1.03	1.50 ± 1.0	5.9 ± 1.3‡	0.002

Data are means ± SE. Analyses were adjusted for ethnicity and sex. The *P* values are by ANOVA. *Genotype frequencies are in Hardy-Weinberg equilibrium; †visceral abdominal tissue (VAT; measured by multislice CT scanning) was adjusted for fatness, ethnicity, and sex; ‡whites only, adjusted for sex; §significantly different from C/C and C/G genotypes. AU, arbitrary unit.

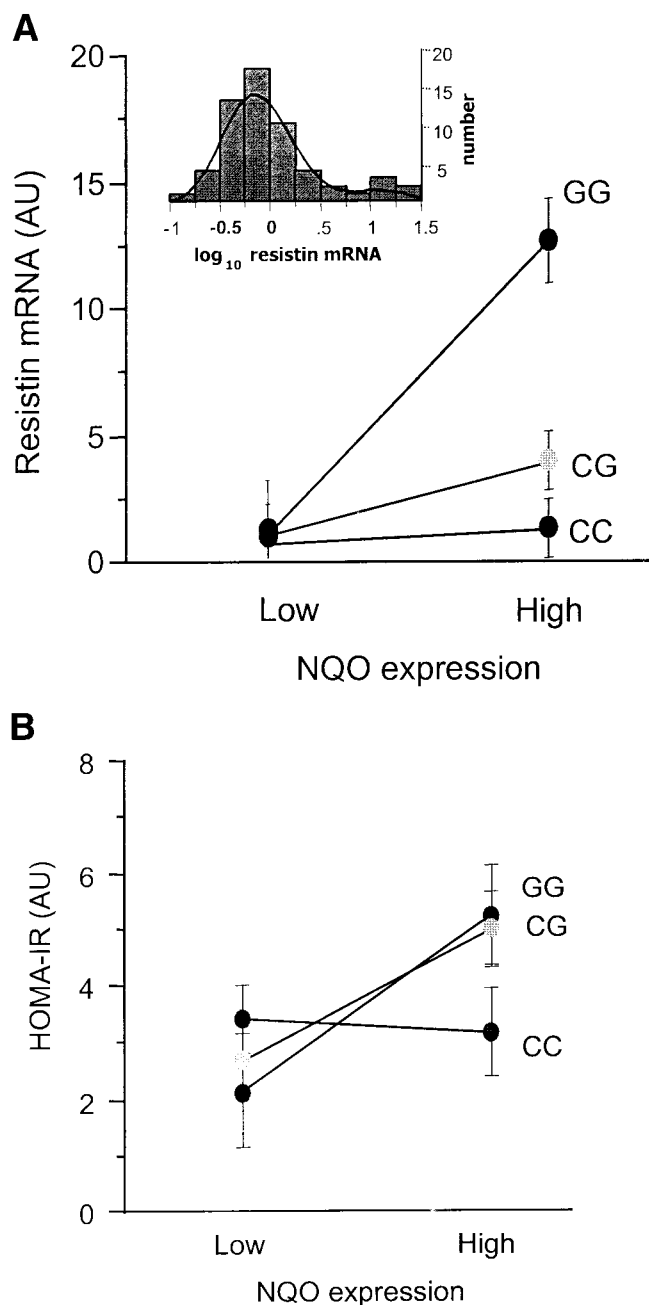


FIG. 2. Interactions of the $-180\text{G}>\text{C}$ SNP with resistin mRNA, insulin resistance, and NQO mRNA. Data are presented as LS means from ANOVA. **A:** There were no significant interactions among the genotype, oxidative stress, and resistin mRNA after the dataset was adjusted for ethnicity and sex (genotype, $P = 0.005$; NQO, $P = 0.0008$; NQO \times genotype, $P = 0.08$ by ANOVA), but the interaction was significant in whites only adjusted for sex (genotype, $P = 0.01$; NQO, $P = 0.003$; NQO \times genotype, $P = 0.05$). **B:** There was a significant interaction among the $-180\text{G}>\text{C}$ SNP, oxidative stress, and HOMA-IR after ethnicity and sex were adjusted for (genotype, $P = 0.6$; NQO, $P = 0.004$; NQO \times genotype, $P = 0.03$), and the interaction remained significant in whites only adjusted for sex (genotype, $P = 0.46$; NQO, $P = 0.005$; NQO \times genotype, $P = 0.04$).

resistin mRNA (genotype, $P = 0.01$; NQO, $P = 0.003$; NQO \times genotype, $P = 0.05$).

Interactions among the $-180\text{G}>\text{C}$ SNP, oxidative stress, and insulin resistance. We next tested the hypothesis that the SNP and oxidative stress interacted with respect to insulin resistance (measured by HOMA-IR) in cohort 2 (Fig. 2B). There was a significant relationship

among the $-180\text{G}>\text{C}$ SNP, oxidative stress, and HOMA-IR (genotype, $P = 0.6$; NQO, $P = 0.004$; NQO \times genotype, $P = 0.03$). Because of the presence of two ethnic groups and sexes in the dataset (cohort 2), data were subsequently adjusted using ethnicity and sex as covariates (Fig. 2B). The interactions among genotype, NQO mRNA, and HOMA-IR remained significant (genotype, $P = 0.72$; NQO, $P = 0.004$; genotype \times NQO, $P = 0.03$). The same analysis was performed for whites only (adjusted for sex) with essentially the same results (genotype, $P = 0.46$; NQO, $P = 0.005$; NQO \times genotype, $P = 0.04$).

Resistin mRNA is related to insulin resistance and hepatic fat accumulation. To test the hypothesis that high resistin mRNA (irrespective of genotype) was related to the insulin resistance syndrome and ectopic fat storage, we constructed multivariate models with insulin resistance (as measured by HOMA-IR) and the amount of liver fat (as measured by the liver:spleen ratio) as dependent variables in cohort 2. We found that high resistin expression was proportionally related to insulin resistance ($P = 0.003$) and to ectopic/hepatic fat ($P = 0.008$), after adjusting for the effects of body fat and visceral adiposity. This effect was present after applying to the data additional adjustments for ethnicity and sex in the case of HOMA-IR ($P = 0.02$; Fig. 3A) and liver fat ($P = 0.008$; Fig. 3B). The same analysis was also performed in whites only, adjusted for sex, and the effect remained significant for both measures (HOMA-IR, $P = 0.01$; liver fat, $P = 0.003$).

DISCUSSION

A functional SNP affects promoter activity in vitro. A functional SNP, $-180\text{G}>\text{C}$, was identified in the promoter of the resistin gene. The polymorphic region had significant promoter activity, which parallels the findings reported for the mouse orthologous region where the proximal 264 nt of the mouse resistin promoter were sufficient for expression of the gene in adipocytes possibly through binding of the adipogenic transcription factor C/EBP α (28). Transfection of differentiated 3T3-L1 cells with the two polymorphic constructs showed that the G/G genotype had fourfold higher promoter activity than the C/C genotype.

$-180\text{G}>\text{C}$ SNP and obesity phenotypes. The possible association of the $-180\text{G}>\text{C}$ SNP with obesity/diabetes phenotypes was examined in a cohort of 978 individuals (cohort 1). G/G homozygous male individuals (i.e., individuals with the high promoter activity genotype) were significantly more obese, by BMI and percentage body fat, than allosygotes, but the significance was eliminated after ethnicity and multiple comparisons were adjusting for. A 5' variant in human resistin, $-420\text{C}>\text{G}$, was recently reported to be associated with obesity (35). This variant is the same as our $-180\text{G}>\text{C}$. The discrepancy in the numbering relates to the numbering schema; they counted as nucleotide -1 the one upstream of the "ATG" translation initiator, whereas we counted as nucleotide -1 the one upstream of the transcription start site. Engert et al. (35) presented significant associations of the "G" allele (i.e., the combined C/G and G/G genotypes) with increased BMI, body weight, body fat mass, and waist circumference, again in men only. This is consistent with our unadjusted-for-multiple-comparisons findings (although in our cohort,

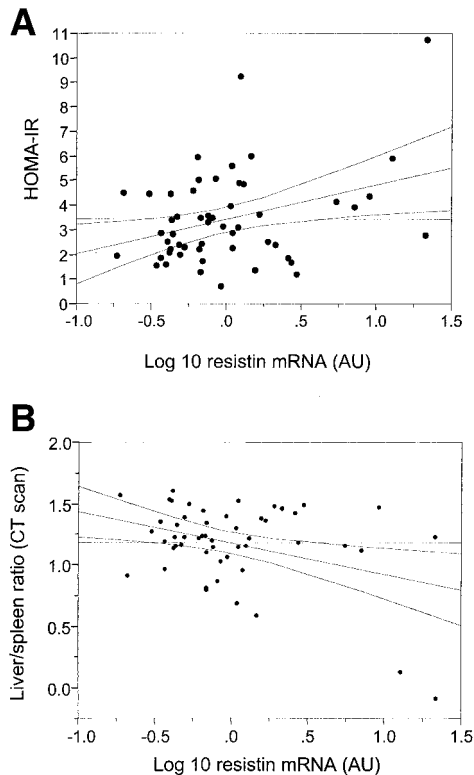


FIG. 3. Resistin mRNA levels are related to insulin resistance and ectopic fat deposition. Data are presented as LS means from ANOVA. **A:** After the effects of body fatness, visceral adiposity, ethnicity, and sex were adjusted for, resistin mRNA in abdominal subcutaneous tissue was proportionally and positively correlated with insulin resistance as measured by HOMA-IR ($P = 0.003$) or when the analysis was performed in whites only adjusted for sex ($P = 0.01$). **B:** After the effects of body fatness, visceral adiposity, ethnicity, and sex were adjusted for, resistin mRNA in abdominal subcutaneous fat was proportionally and positively correlated with fat accumulation in the liver, measured as the liver:spleen ratio ($P = 0.008$). This correlation remained significant when the analysis was performed in whites only adjusted for sex ($P = 0.003$).

the G/G genotype had a significant effect on its own), but these data do not represent conclusive evidence for an association with obesity. Two additional publications have also reported the $-180C>G$ SNP (referred to as $-179C>G$ SNP) but did not find any associations of this SNP with obesity (36,37) or in linkage disequilibrium with other SNPs in the gene. Taken together the findings of the four reports (35–37), there is an overall consensus that the $-180C>G$ SNP is not associated with obesity (when considering each genotype on its own), but there may be populations or yet-to-be-determined circumstances in which the G/G genotype might predispose for higher percentage body fat.

A functional role for the $-180C>G$ SNP in vivo. Because the in vitro data suggested that the $-180C>G$ SNP might affect expression of resistin, we tested this hypothesis in a cohort of 58 overweight and hyperinsulinemic individuals (cohort 2) by measuring resistin mRNA expression in abdominal subcutaneous adipose tissue. The data set was adjusted for ethnicity and sex, and no effects of the three genotypes were observed on BMI, fasting glucose, the liver:spleen ratio, fat mass, insulin levels, HOMA-IR, and visceral adiposity. In contrast, G/G homozygotes (i.e., individuals with the high promoter activity genotype) had significantly higher expression levels of

resistin than allozygotes, confirming our hypothesis and recapitulating the in vitro data. This analysis was repeated for whites only, and G/G homozygotes still had significantly higher resistin mRNA levels compared with allozygotes. The association of the G/G genotype with high resistin mRNA remained significant even after multiple testing was adjusted for. Further experiments to determine the protein levels of resistin would be required to investigate whether the higher resistin mRNA levels in G/G homozygotes also translate to higher protein levels, thus confirming the physiological functionality of the SNP. For several individuals with high resistin mRNA, we measured resistin in a second adipose tissue sample separated from the first one by 8 weeks. Resistin mRNA was similar in the two samples (data not shown). As such, resistin mRNA expression is stable over time and the high expression in G/G homozygotes is not a transient event. However, although all C/C homozygotes had low resistin mRNA, not all G/G homozygotes exhibited high resistin mRNA. This was reflected by the bimodal distribution of resistin mRNA levels in cohort 2 (Fig. 2A, inset). We therefore hypothesized that there was an additional factor involved in the regulation of resistin other than the $-180C>G$ genotype.

Interactions among the $-180C>G$ SNP, oxidative stress, and resistin mRNA/HOMA-IR. To test the hypothesis that oxidative stress was the additional factor involved in the regulation of resistin, we measured the effects of oxidative stress on resistin mRNA in vivo. Our rationale for measuring oxidative stress was based on the fact that previous studies have demonstrated a role for oxidative stress as an activator of the Sp-1 transcription factor, one of the transcription factors that might bind to the -180 GC SNP (38,39) and the putative role of oxidative stress in insulin resistance (reviewed by Evans et al. [23]). Oxidative stress was measured by the expression levels of NQO, a prototypical phase II antioxidant enzyme. In addition, NQO knockout mice exhibit alterations in the intracellular redox state and have reduced visceral adipose tissue mass (22).

There was no direct correlation between the $-180C>G$ SNP and NQO mRNA. The causes of high oxidative stress in human fat cells could be the result of a number of factors, including high-fat diet, inactivity, smoking, and so forth. Analyses of the data showed a significant interaction among the $-180C>G$ SNP, NQO mRNA, and resistin mRNA, but this interaction did not hold significance after ethnicity and sex were adjusted for, which could be due to the disproportionate frequencies of the genotype in the black individuals of this cohort. This possibility was confirmed when the white individuals only were examined (adjusted for sex), where the interaction among genotype, resistin mRNA, and NQO mRNA was significant.

The interaction among the $-180C>G$ SNP, NQO mRNA, and HOMA-IR was also tested to examine for possible effects of the genotype on insulin resistance with relation to the levels of oxidative stress. There was a significant interaction illustrating an effect of the SNP alone and oxidative stress only when the GG genotype was present. These data suggest that the resistin $-180C>G$ SNP and cellular oxidative stress are potentially related to the insulin resistance syndrome in *Homo sapiens*.

Resistin is directly and independently related to insulin resistance and liver fat. We next tested the hypothesis that resistin mRNA levels (irrespective of genotype) were directly related to insulin action. We found that high resistin expression was proportionally related to insulin resistance, after adjusting for the effects of body fat and visceral adiposity. This effect was present after we adjusted further for ethnicity and sex and when we performed the analysis in white individuals only (also adjusted for sex).

Fatty liver is also a component of the insulin resistance syndrome (32) and contributes to increased hepatic glucose output. To test the relationship of resistin mRNA to fatty liver, we constructed a multivariate model with the amount of liver fat as the dependent variable and adjusted for the major effects of fatness and visceral adiposity. We found that resistin mRNA was directly and independently related to liver fat infiltration.

CONCLUSIONS

There are two putative roles of resistin: 1) to directly cause insulin resistance (as proposed by Lazar et al. [40]) and 2) to block adipocyte differentiation (as proposed by Sul et al. [14]). The latter might lead to ectopic fat storage (i.e., increased amounts of fat in skeletal muscle and liver; reviewed in Smith and Ravussin [41]). Here we report that the G/G genotype of an SNP in the promoter of the human resistin gene, $-180C>G$, had significantly increased basal promoter activity in adipocytes. These data were recapitulated in vivo where G/G homozygotes had significantly higher resistin mRNA levels in human abdominal subcutaneous fat. A significant interaction was also found among the $-180C>G$ SNP, a marker of oxidative stress (NQO), and insulin resistance (HOMA-IR). In addition, resistin mRNA was positively and independently correlated with insulin resistance and hepatic fat as measured by liver X-ray attenuation. These data potentially implicate resistin in the pathophysiology of the human insulin resistance syndrome, an effect mediated by the $-180C>G$ promoter SNP and, possibly, cellular oxidative stress.

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