Endoplasmic Reticulum Stress Regulates Adipocyte Resistin Expression

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OBJECTIVE—Resistin is a secreted polypeptide that impairs glucose metabolism and, in rodents, is derived exclusively from adipocytes. In murine obesity, resistin circulates at elevated levels but its gene expression in adipose tissue is paradoxically reduced. The mechanism behind the downregulation of resistin mRNA is poorly understood. We investigated whether endoplasmic reticulum (ER) stress, which is characteristic of obese adipose tissue, regulates resistin expression in cultured mouse adipocytes.

RESEARCH DESIGN AND METHODS—The effects of endoplasmic stress inducers on resistin mRNA and secreted protein levels were examined in differentiated 3T3-L1 adipocytes, focusing on the expression and genomic binding of transcriptional regulators of resistin. The association between downregulated resistin mRNA and induction of ER stress was also investigated in the adipose tissue of mice fed a high-fat diet.

RESULTS—ER stress reduced resistin mRNA in 3T3-L1 adipocytes in a time- and dose-dependent manner. The effects of ER stress were transcriptional because of downregulation of CAAT/enhancer binding protein-α and peroxisome proliferator–activated receptor-γ transcriptional activators and upregulation of the transcriptional repressor CAAT/enhancer binding protein homologous protein-10 (CHOP10). Resistin protein was also substantially downregulated, showing a close correspondence with mRNA levels in 3T3-L1 adipocytes as well as in the fat pads of obese mice.

CONCLUSIONS—ER stress is a potent regulator of resistin, suggesting that ER stress may underlie the local downregulation of resistin mRNA and protein in fat in murine obesity. The paradoxical increase in plasma may be because of various systemic abnormalities associated with obesity and insulin resistance. *Diabetes* **58**: 1879–1886, 2009

The growing obesity epidemic and the comorbidities associated with it, including insulin resistance, cardiovascular disease, and cancer, have made adipose tissue an important subject of scientific study and a target of therapeutic interventions. In addition to being a storage depot of excess energy, adipose tissue is an active endocrine organ that secretes unique proteins known as adipokines such as adiponectin, leptin, and resistin. Under physiologic conditions, adipokines contribute to the maintenance of whole-body glucose homeostasis, for example, by modulating gluconeogenesis in the liver or energy expenditure and appetite in the brain (1). In obesity, however, their expression is dysregulated, leading to various metabolic abnormalities, including hyperglycemia and hyperlipidemia, which in turn contribute to insulin resistance and heart disease (2,3). Therefore, understanding how adipokine expression is regulated under physiologic and pathologic conditions is critical to the ability to therapeutically modulate their action in the future (1,4).

One adipokine that contributes to insulin resistance in mouse models of obesity is resistin. Resistin is exclusively made by adipocytes in mice (5), and its serum levels increase as obesity develops (6,7). Although resistin is produced by macrophages in humans rather than adipocytes (8) and its role in human obesity is controversial (9), a number of clinical studies have linked elevated serum resistin levels with cardiovascular disease (10–12), implicating resistin in metabolic disease in humans as well as in mice. Importantly, resistin-deficient mice have improved glucose tolerance compared with wild-type controls both in diet-induced obesity (5) and in leptin deficiency (13), suggesting a role for resistin in insulin resistance. Loss-of-function and gain-of-function studies have demonstrated that resistin modulates liver glucose production through decreased activation of AMP-activated protein kinase (AMPK) and increased expression of gluconeogenic enzymes (5,13–16). Several recent studies suggest that resistin may act centrally in the hypothalamus to regulate glucose homeostasis (17,18).

There is increasing evidence that in obese individuals adipose tissue experiences different types of stress including inflammation, hypoxia, oxidative stress, metabolic stress from overabundance of nutrients, and mechanical stress from hypertrophy (19,20). Recently, Hotamisligil and colleagues have demonstrated that adipose tissue from obese mice shows signs of an activated endoplasmic reticulum (ER) stress response (21). Although the exact etiology of ER stress in obese adipose tissue is unknown, it may result from nutrient overload, an increased demand for protein synthesis, or local glucose deprivation in the setting of insulin resistance and decreased adipose tissue vascularization (19). Therefore, unresolved ER stress may contribute to the dysregulated function of adipose tissue by diminishing insulin sensitivity and leading to aberrant adipokine secretion (19).

A curious aspect of resistin biology is that, despite rising serum levels, resistin mRNA levels are significantly decreased in adipose tissue in obese mouse models (15,22,23). Endoplasmic reticulum stress was recently shown to downregulate adiponectin (24), and we hypothesized that the decrease in resistin mRNA seen in obese

adipose tissue may be a result of activation of the ER stress response. We show that induction of ER stress in vitro can lead to downregulation of resistin mRNA levels in a time- and dose-dependent manner, and the mechanism appears to be transcriptional. This effect involves changes in the levels of several transcriptional regulators of resistin: enhancer binding protein-α (C/EBPα), peroxisome proliferator–activated receptor (PPAR)γ, and CAAT/enhancer binding protein homologous protein-10 (CHOP10). Altering the levels of these transcription factors mimics or partially rescues the effects of ER stress on resistin expression. We have uncovered a previously unknown link between activation of the ER stress in mouse adipocytes and resistin, which may be of significance in vivo in the setting of obesity.

RESEARCH DESIGN AND METHODS

Six-week-old wild-type male C57Bl/6 mice were placed on high-fat diet (HFD) from Research Diets (45 kcal% fat) or normal chow (NC) (6 kcal% fat) for 30 weeks. At the end of the study, blood samples for serum resistin measurement were collected. Epididimal white adipose tissue was isolated and processed for RNA or protein. All animal experiments were performed at the University of Pennsylvania according to protocols approved by the Institutional Animal Care and Use Committee.

Cell culture and treatment. 3T3-L1 preadipocytes (American *Type Culture* Collection) were cultured in growth medium (high-glucose Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% FBS (U.S. Biotechnologies) and 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Two days postconfluence, the cells were induced to differentiate with standard cocktail consisting of growth medium with 1 μmol/l dexamethasone, 10 μg/ml bovine insulin, and 0.5 mmol/l isobutyl-1-methylxanthine (Sigma). After 3 days in differentiation medium, the cells were treated with growth medium with 10 μg/ml bovine insulin for 2 days and then maintained in growth medium alone. Cells were considered mature adipocytes 8 days postinduction of differentiation, when knockdown experiments or treatment with tunicamycin, thapsigargin, or actinomycin D (all from Sigma) were performed.

Short interfering RNA oligo transfection. Short interfering RNA (siRNA) oligos targeting C/EBPα, PPARγ, CHOP10, and resistin (L-051631-00), as well as a nontarget negative control, were obtained from Dharmacon. Transfection was performed by electroporation with Nucleofector II and Cell Line Nucleofector Kit V (AMAXA). Electroporated cells were reseeded in growth medium and harvested or used for treatment at 24 h or 48 h post-transfection. Sense sequences for the specific target oligos are as follows:

    C/EBPα_1 GAGCCGAGAUAAAGCCAAAUU
    C/EBPα_2 CCUGAGAGCUCCUUGGUCAUU
    C/EBPα_3 GGAGUUGACCAGUGACAAUUU
    C/EBPα_4 CUAUAGACAUCAGCGCCUAUU
    CHOP10_1 CAACAGAGGUCACACGCACUU
    CHOP10_2 GCACCAAGCAUGAACAGUGUU
    CHOP10_3 GAGCAAGGAAGAACUAGGAUU
    CHOP10_4 GAAACAGAGUGGUCAGUGCUU
    PPARγ CAACAGGCCUCAUGAAGAAUU

Luciferase assays. Resistin-luc was generated by inserting −13580/+243 bp fragment of the mouse resistin promoter/enhancer into pGL3-basic vector (Promega) as described previously (31). 3T3-L1 adipocytes were transfected by electroporation with Nucleofector II (AMAXA). Briefly, mature adipocytes (day 10 after differentiation) were detached from culture dishes with 0.25% trypsin, washed twice with 1× PBS, and resuspended in electroporation buffer (solution V, AMAXA). Approximately 1×10^6 were electroporated (electroporation program T-020) with 2 μg of pGL3-basic or resistin-luc and 0.3 μg of β-galactosidase expression vector and seeded into 3 wells of a 24-well plate. After 16 h, the medium was replaced with fresh culture medium with vehicle or 5 μg/ml tunicamycin. The cells were incubated for 24 additional hours, and luciferase activity was measured by a luciferase assay kit (Promega). Light units were normalized to β-galactosidase activity. Fold activations relative to the pGL3-basic and vehicle were calculated, and the results of triplicate samples were plotted.

Resistin protein measurements. Resistin levels in tissue culture media, whole cell lysates, and EWAT lysates (homogenized in PBS) were measured using a mouse resistin ELISA (Millipore) following the manufacturer's instructions. Samples were diluted appropriately before loading. Serum resistin levels in the mouse diet-induced obesity study were measured using a mouse

Adipokine LINCOplex Assay (Millipore) according to the manufacturer's instructions. Total cell protein was measured using diluted samples on a NanoDrop Spectrophotometer (Thermo Scientific).

RNA isolation and quantitative PCR. RNA was isolated from cells with the RNeasy Mini Kit or from adipose tissue with the RNeasy Lipid kit (both from Qiagen). Reverse transcription of ~0.5 μg of RNA was performed with the RT FOR PCR ADVANTAGE KIT (Clontech) following the manufacturer's instructions. Quantitative PCR (QPCR) was performed using TaqMan Polymerase Universal Master Mix or Power SYBR Green PCR Mastermix (Applied Biosystems) and the PRISM 7500 instrument (Applied Biosystems). Data were analyzed using the standard curve method and normalization of all genes of interest to the house-keeping control gene Arbp/36b4. Analysis of activating transcription factor 3 (ATF3), PPARγ, mouse, and human C/EBPα expression was conducted using TaqMan Gene Expression Assays (Applied Biosystems). Primer sequences used for QPCR analysis of CHOP10 and BiP mRNA were obtained from Rutkowski et al. (25). The remaining primer sequences are as follows:

    F-resistin: TCATTTCCCCTCCTTTTCCTTT
    R-resistin: TGGGACACAGTGGCATGCT
    F-Arbp/36b4: CAACCCAGCTCTGGAGAAAC
    R-Arbp/36b4: CCAACAGCATATCCCGAATC

Immunoblotting. Cell protein extracts were generated in cold whole-cell extract buffer (0.15 M NaCl, 0.05 M Tris, pH 7.4, 0.005 M EDTA, 0.5% NP-40) with Complete protease inhibitors (Roche). Immunoblotting following SDS-PAGE was performed using the following antibodies: anti-C/EBPα (sc-61, Santa Cruz), anti-PPARγ (sc-7273, Santa Cruz), anti-HDAC2 (sc-7899, Santa Cruz), and anti-Ran (610341, BD Biosciences), anti-phospho-eIF2α (Ser51) (119A11, Cell Signaling), total eIF2α (sc-11386, Santa Cruz).

Chromatin immunoprecipitation. Cross-linking of adipocyte proteins and chromatin was performed in 1% Formaldehyde (Sigma), followed by quenching in 125 mmol/l glycine and two washes with 1× PBS (Invitrogen). Nuclear pellets were obtained following dounce homogenization in nuclear lysis buffer (20 mmol/l HEPES, 0.25 M sucrose, 3 mmol/l MgCl₂, 0.2% NP-40, 3 mmol/l β-mercaptoethanol, 0.4 mmol/l phenylmethylsulfonyl fluoride, complete protease inhibitor tablets). Sonication was carried out with Bioruptor (Diagenode) in chromatin immunoprecipitation (ChIP) SDS lysis buffer (50 mmol/l HEPES, 1% SDS, 10 mmol/l EDTA). Immunoprecipitations were performed using ~100 μg/ml chromatin and 10 μg of antibody: anti-C/EBPα (sc-61, Santa Cruz), anti-PPARγ (sc-7196, Santa Cruz), or nonspecific rabbit IgG control (Sigma). After cross-link reversal, DNA was isolated with phenol/chloroform/isoamyl alcohol, and enrichment was measured by QPCR, using Power SYBR Green PCR Mastermix (Applied Biosystems) and the PRISM 7500 instrument (Applied Biosystems). Analysis was performed by the standard curve method and normalization to a nontarget control region of the Arbp/36b4 gene. Primers used for QPCR analysis are as follows:

    F-dnstr-resistin-50bp TCCCTCCTCTGGGACCTCTA
    R-dnstr-resistin-50bp CCCATCCTGCCTTGGATAAT
    F-upstr-resistin-9kb GTAAGGGGGTGGCCTGATAG
    R-upstr-resistin-9kb ATTCCCTTCTCCCACCAAGT
    F-Arbp/36b4 CTGGGACGATGAATGAGGAT
    R-Arbp/36b4 AGCAGCTGGCACCTAAACAG
    F-ins1 CTTCAGCCCAGTTGACCAAT
    R-ins1 AGGGAGGAGGAAAGCAGAAC
    F-albumin CTTCAGCCCAGTTGACCAAT
    R-albumin AGGGAGGAGGAAAGCAGAAC

Statistical analysis. Student's *t* test was used to determine *P* values.

RESULTS

Resistin is downregulated by ER stress induction in 3T3-L1 adipocytes. To investigate the potential link between ER stress and resistin downregulation, mouse 3T3-L1 adipocytes were incubated with thapsigargin, which causes ER stress by inhibiting the sarco/ER Ca²⁺ pump, and led to dramatically reduced resistin mRNA levels (Fig. 1*A*). To confirm ER stress as the mechanism underlying resistin downregulation, adipocytes were treated with tunicamycin, which induces ER stress by inhibiting *N*-linked glycosylation of newly synthesized proteins. Tunicamycin treatment markedly downregulated resistin mRNA levels in a time-dependent fashion
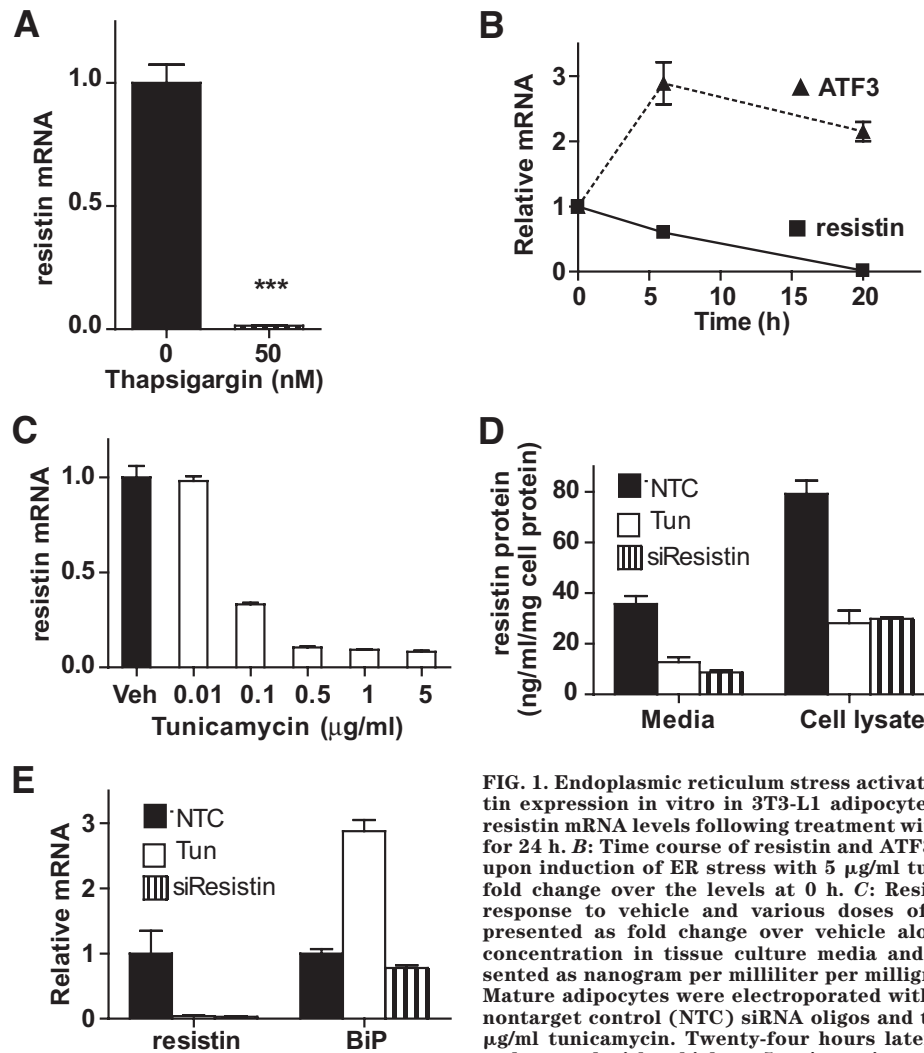
FIG. 1. Endoplasmic reticulum stress activation downregulates resistin expression in vitro in 3T3-L1 adipocytes. *A*: Downregulation of resistin mRNA levels following treatment with 50 nmol/l thapsigargin for 24 h. *B*: Time course of resistin and ATF3 mRNA gene expression upon induction of ER stress with 5 μg/ml tunicamycin, presented as fold change over the levels at 0 h. *C*: Resistin gene expression in response to vehicle and various doses of tunicamycin for 24 h, presented as fold change over vehicle alone. *D*: Resistin protein concentration in tissue culture media and whole cell lysates presented as nanogram per milliliter per milligram of total cell protein. Mature adipocytes were electroporated with resistin (siResistin) or nontarget control (NTC) siRNA oligos and treated with vehicle or 5 μg/ml tunicamycin. Twenty-four hours later the cells were washed and treated with vehicle or 5 μg/m tunicamycin for 24 h and resistin protein levels were assayed with ELISA. Data are mean ± SE, *n* = 3. *E*: Gene expression validation that siResistin and tunicamycin treatment reduced resistin mRNA to similar levels, and only tunicamycin induced markers of ER stress such as BiP. Data in *A–C* and *E* were normalized to the house-keeping gene Arbp/36b4 and are shown as mean ± SE, *n* = 3. ***$P < 0.001$.

(Fig. 1*B*). By contrast, the same treatment induced expression of ATF3, which is activated by ER stress (26). The decrease of resistin mRNA by tunicamycin was also dose-dependent (Fig. 1*C*). To examine the effects of tunicamycin on resistin protein levels, cells were treated for 24 h to lower resistin mRNA as in Fig. 1*C*, then treated with fresh tunicamycin- or vehicle-containing media, after which the accumulation of secreted resistin as well as the intracellular resistin levels were measured by ELISA. Resistin protein was substantially decreased in the media and cell lysate of tunicamycin-treated cells (Fig. 1*D*), similar to that observed with resistin knockdown, which reduced the mRNA to similar extent but did not induce upregulation of BiP mRNA that would have signified ER stress (Fig. 1*E*). Finally, the effects of tunicamycin on resistin secretion also appeared to be dose dependent (data not shown). Collectively, these results indicate that ER stress is a potent regulator of resistin mRNA and protein levels in 3T3-L1 adipocytes.

Activation of ER stress in white adipose tissue of obese mice is associated with reduced levels of both mRNA and tissue protein of resistin. It has been reported previously that in obese mice, resistin mRNA levels are decreased while protein levels in the circulation are increased compared with lean mice, raising the possibility that during obesity there is dissociation between resistin mRNA and protein levels. A reasonable prediction, then, would be that protein levels in the fat pad may also be higher in obese versus lean mice, similar to what is seen in the circulation. To address this question, C57Bl/6 mice were fed HFD or NC for 30 weeks, at which point resistin mRNA levels were decreased in epididymal white adipose tissue (EWAT) of the HFD mice (Fig. 2*A*), despite increased serum resistin levels (Fig. 2*B*). Surprisingly, EWAT resistin protein levels were not increased, but rather tended to be lower in the HFD mice (Fig. 2*C*). This indicates that locally in the fat pad, resistin protein levels correspond to the mRNA changes, similar to what was observed in the tunicamycin-treated cells. Furthermore, consistent with previous reports of ER stress in obesity (21,27), markers of ER stress such as phospho-eIF2α (Fig. 2*D*) and BiP mRNA levels (Fig. 2*E*) were elevated in the adipose tissue from the HFD-fed obese mice. Taken to-

A
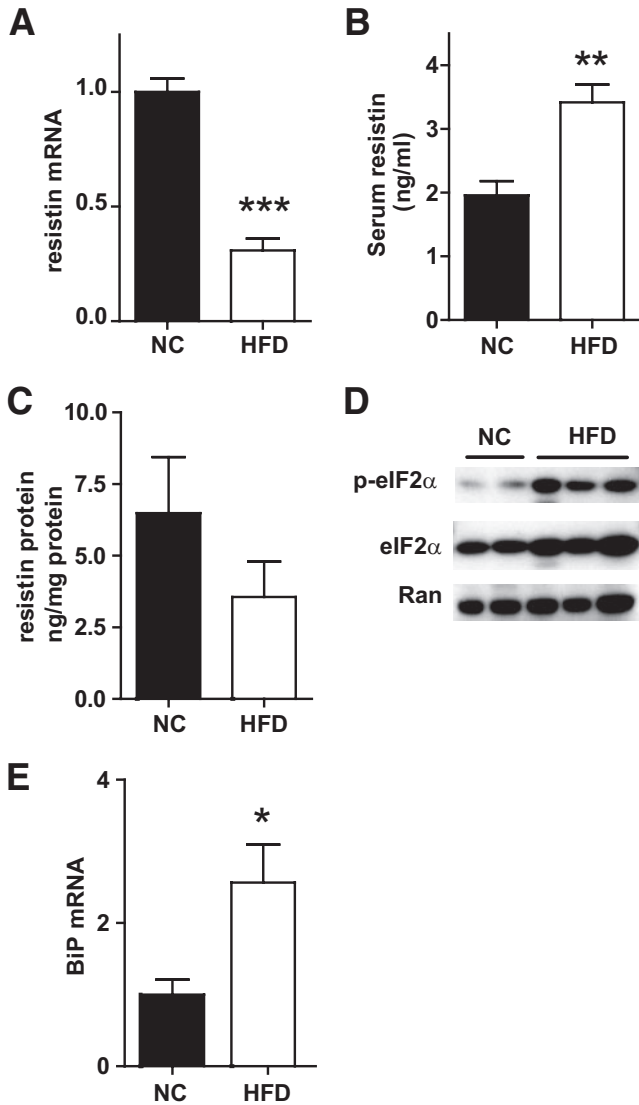

B

C

D

E

FIG. 2. Reduced resistin levels in EWAT of obese mice are associated with markers of ER stress. All data are from male C57Bl/6 mice fed NC ($n = 5$) or HFD ($n = 5$) for 30 weeks to induce obesity. *A*: Resistin mRNA levels in EWAT. Data were normalized to the house-keeping gene Arbp/36b4 and presented as mean ± SE. *B*: Serum resistin levels after 30 weeks of diet. *C*: Resistin protein levels in EWAT, measured with ELISA in tissue homogenates, and normalized to the total protein concentration of each homogenate. Data are presented as nanogram resistin per milligram total protein, mean ± SE. *D*: Western blot demonstrating the phosphorylation status of Eukaryotic translation initiation factor 2a (eIF2α) in representative animals selected at random from the NC and HFD groups. Total eIF2α and Ran were used as loading controls. *E*: mRNA levels of the ER chaperone BiP measured in EWAT. Data were normalized to the house-keeping gene Arbp/36b4 and are presented as mean ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.
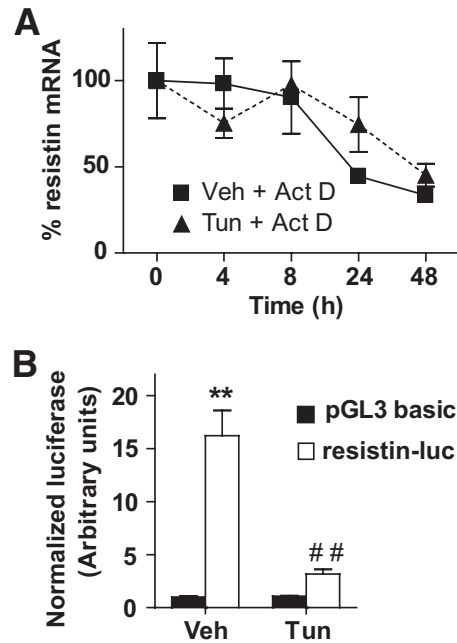
A

B

FIG. 3. Endoplasmic reticulum stress downregulates resistin in adipocytes by a transcriptional mechanism. *A*: Changes in resistin mRNA levels over 48 h in response to vehicle or 5 μg/ml tunicamycin in the presence of 5 μg/ml Actinomycin D. Data are presented as percent of the mRNA level at time 0, mean ± SD of a triplicate experiment. *B*: Normalized luciferase activity of resistin-luc ($-13,580$ bp to $+243$ bp) or empty vector (pGL3 basic) in the presence of vehicle or 5 μg/ml tunicamycin for 24 h. Data are presented as mean ± SE, $n = 3$. **$P = 0.003$ for pGL3 basic versus resistin-luc activity in vehicle-treated cells. ##$P = 0.005$ for resistin-luc activity in vehicle- versus tunicamycin-treated cells.

would have been expected if ER stress reduced resistin mRNA by a post-transcriptional mechanism (Fig. 3*A*). The effect of ER stress was further explored using a luciferase reporter vector (resistin-luc) driven by a large fragment of the resistin gene including the promoter and transcriptional start site ($-13,580$ bp to $+243$ bp). The resistin-luc reporter was active in mature adipocytes, but most of this activity was lost when the cells were treated with 5 μg/ml tunicamycin for 24 h (Fig. 3*B*), further demonstrating that ER stress causes reduced resistin gene transcription.

Downregulation of C/EBPα contributes to the decrease in resistin mRNA by tunicamycin. One candidate transcription factor that could explain the effects of ER stress on resistin expression is C/EBPα. C/EBPα is critical for adipocyte differentiation (28) and contributes to activation of adipocyte-specific genes such as adiponectin (29), PPARγ (30), and resistin (31). A binding site for C/EBPα on the resistin promoter has been characterized, and it has been shown that ectopic C/EBPα expression in nonadipogenic cells could drive luciferase expression from the resistin promoter (31). Furthermore, C/EBPα was recently reported to be downregulated on the mRNA level by the inducers of ER stress tunicamycin and thapsigargin (32), and indeed we confirmed that C/EBPα mRNA and protein are reduced in tunicamycin-treated adipocytes (Fig. 4*A* and *B*). This treatment also abolished C/EBPα occupancy at the resistin gene as measured by ChIP both at the previously characterized C/EBPα binding site (31) as well as an additional upstream site identified in a recent genome-wide ChIP-chip study (33) (Fig. 4*C*). Similar decreases in occupancy were noted on the known C/EBPα
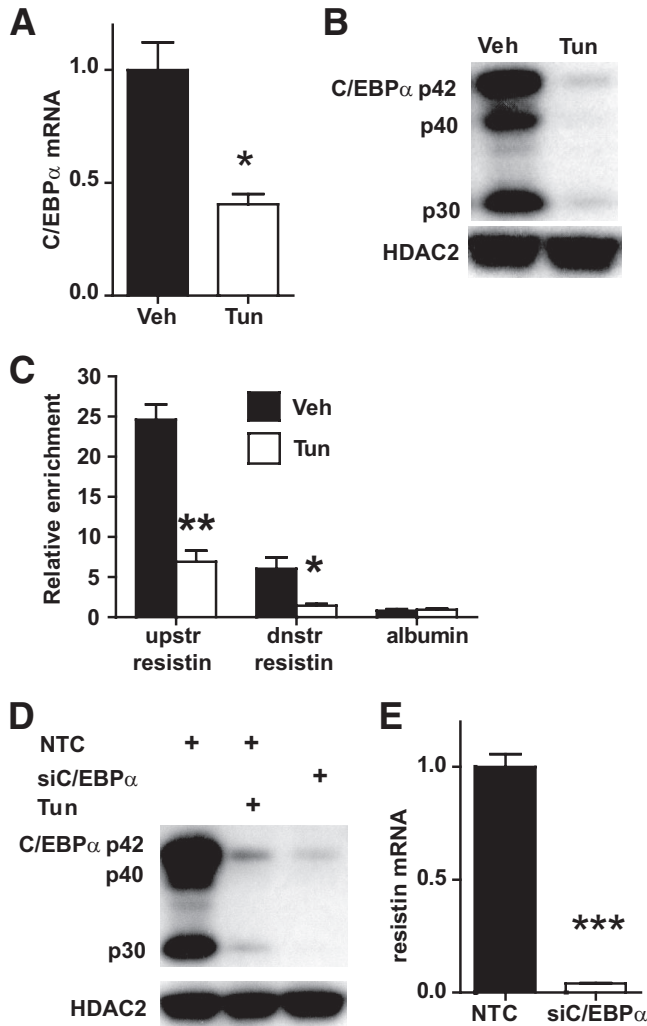
gether, these data suggest that ER stress may be a relevant mechanism in the downregulation of resistin in vivo in the setting of mouse obesity.

Endoplasmic reticulum stress downregulates resistin mRNA by a transcriptional mechanism. An initial step in dissecting the mechanism by which ER stress regulates resistin expression was to determine whether the downregulation of resistin by tunicamycin is transcriptional. For this purpose, 3T3-L1 adipocytes were treated with 5 μg/ml tunicamycin in the presence of 5 μg/ml of the transcriptional inhibitor Actinomycin D. Tunicamycin treatment did not reduce the half-life of resistin mRNA as
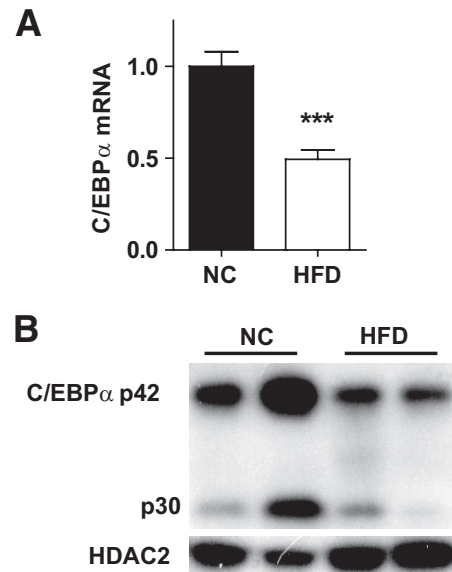
FIG. 5. C/EBPα is downregulated in EWAT of obese mice. All data were derived from EWAT of the same animals as in Fig. 1, NC ($n = 5$) or HFD ($n = 5$). *A*: C/EBPα mRNA levels normalized to Arbp/36b4 and are presented as mean ± SE. *B*: C/EBPα protein levels were measured by Western blotting in EWAT of two randomly selected animals per group. HDAC2 was used as a loading control. ***$P < 0.001$.

FIG. 4. Downregulation of C/EBPα by ER stress decreases resistin expression. *A* and *B*: Endoplasmic reticulum stress induced by treatment with 5 μg/ml tunicamycin for 24 h leads to downregulation of C/EBPα mRNA and protein compared with vehicle control. Gene expression data are presented as mean ± SE, $n = 3$. C/EBPα protein levels were assayed by Western blotting, and HDAC2 served as a loading control. *C*: Endoplasmic reticulum stress reduces C/EBPα recruitment to resistin at a downstream site at the known C/EBPα binding site at −50 bp relative to the transcription start site (TSS), and at a site located ~9 kb upstream of the TSS. Mature adipocytes were treated as in *A*, and recruitment was measured by ChIP-QPCR. A region at the TSS of albumin was used as negative control for C/EBPα recruitment. Enrichment was normalized to a site on the Arbp/36b4 gene where C/EBPα does not bind. Data are presented as mean ± SE of three independent ChIP experiments. *D*: C/EBPα knockdown (siC/EBPα) mimics the effects of ER stress on resistin expression. Mature adipocytes, electroporated with C/EBPα or nontarget control (NTC) siRNA oligos, were treated with vehicle or 5 μg/m tunicamycin for 24 h. Efficiency of siC/EBPα was assayed by Western blot and compared with NTC in the presence or absence of 5 μg/m tunicamycin. HDAC2 was used as a loading control. *E*: Resistin mRNA levels were measured in vehicle-treated treated C/EBPα kd or NTC cells by QPCR and normalized to Arbp/36b4. Data are presented as mean ± SE, $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

binding sites on the promoters of PPARγ and adiponectin (data not shown).

Next, we investigated whether knockdown of C/EBPα could recapitulate the effects seen by tunicamycin treatment. For this purpose, mature adipocytes were electroporated with siRNA oligonucleotides against C/EBPα or a nontarget control. This strategy reduced C/EBPα protein to levels similar to those seen in control cells treated with 5 μg/ml tunicamycin (Fig. 4*D*). Resistin mRNA levels,

measured 48 h after electroporation, appeared greatly reduced when C/EBPα had been knocked down (Fig. 4*E*). Interestingly, measurement of C/EBPα mRNA (Fig. 5*A*) and protein (Fig. 5*B*) in WAT of obese mice revealed that C/EBPα levels were also decreased relative to WAT of lean mice. This finding suggests that in vivo as well as in vitro, ER stress activation is associated with decreased C/EBPα levels, which may account for the effects seen on resistin expression. Of note, C/EBPβ levels were not significantly changed in adipocytes by tunicamycin treatment or by HFD feeding in WAT (data not shown).

Decreased PPARγ expression and activity also contribute to the effects of ER stress. Our recent genome-wide study of C/EBP and PPARγ binding in adipocytes (33) demonstrated that PPARγ binds near C/EBPα at the upstream resistin enhancer. PPARγ, which is crucial for adipogenesis (34,35), also induces resistin expression during adipocyte differentiation (36). Therefore, we examined whether changes in PPARγ levels may also mediate the effects of ER stress on resistin expression. Indeed, tunicamycin treatment of adipocytes substantially reduced PPARγ gene expression (Fig. 6*A*) as has been reported previously (24). Moreover, tunicamycin treatment nearly abolished recruitment of PPARγ to the resistin gene (Fig. 6*B*). Furthermore, knockdown of PPARγ in mature adipocytes reduced resistin mRNA levels by ~90% (Fig. 6*C*). Efficiency of PPARγ knockdown was assessed by measuring PPARγ mRNA levels by QPCR (Fig. 6*D*). Thus, PPARγ downregulation contributes to the effects of ER stress on resistin gene expression.

CHOP10 also regulates resistin expression in the setting of ER stress activation. Another transcription factor that is active in adipocytes under conditions of stress and was recently shown to regulate expression of the adipokine adiponectin is CHOP10 (24). This protein bears significant homology to other C/EBPs and is induced by various stressors, including hypoxia and the unfolded protein response (37). Although it does not bind DNA alone, it can have dominant negative interactions with
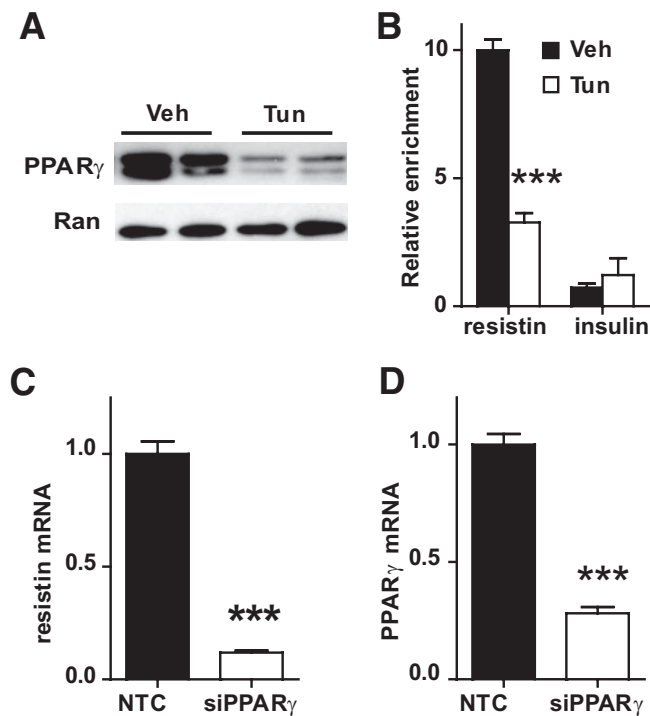
FIG. 6. Downregulation of PPARγ contributes to the effects of ER stress on resistin expression. *A*: PPARγ protein levels were measured by Western blotting in mature adipocytes treated with vehicle or 5 μg/ml tunicamycin for 24 h. Ran was used as a loading control. *B*: Endoplasmic reticulum stress decreases recruitment of PPARγ at a binding site near the resitin gene. ChIP-QPCR was performed as in Fig. 4*C*. A region at the insulin gene was used as negative control for PPARγ recruitment. Data are presented as mean ± SE of three independent ChIP experiments. *C*: PPARγ knockdown (siPPARγ) can mimic the effects of ER stress on resistin. Mature adipocytes were electroporated with PPARγ (siPPARγ) or NTC siRNA oligos. Resistin mRNA levels were measured 48 h later by QPCR and normalized to Arbp/36b4. *D*: Efficiency of PPARγ knockdown was assayed by QPCR. *C* and *D*: Data are presented as mean + SE of three replicates. ***$P < 0.001$.

FIG. 7. Upregulation of CHOP10 by ER stress induction contributes to reduced resistin expression. *A*: CHOP10 is upregulated by ER stress. CHOP10 mRNA levels were measured by QPCR in mature adipocytes treated with vehicle or 5 μg/ml tunicamycin for 24 h. *B–D*: CHOP10 knockdown (siCHOP10) partially rescues the effects of ER stress on resistin and C/EBPα expression. *B*: Efficiency of CHOP10 knockdown was assayed by QPCR. *C*: Effects of siCHOP10 or NTC on resistin mRNA levels in the presence or absence of 5 μg/ml tunicamycin for 24 h. *D*: Effects of siCHOP10 on C/EBPα mRNA levels. All QPCR data were normalized to Arbp/36b4 and presented as mean ± SE of three replicates. nontarget control, NTC. **$P < 0.01$, ***$P < 0.001$.

other C/EBPs leading to decreased transcription of their targets (37,38). As expected from other cell types, CHOP10 gene expression was elevated in the setting of ER stress in 3T3-L1 adipocytes (Fig. 7*A*). Knockdown of CHOP10 (Fig. 7*B*) partially prevented the tunicamycin-induced decrease in adipocyte resistin mRNA (Fig. 7*C*). CHOP10 depletion also increased C/EBPα expression in tunicamycin-treated adipocytes (Fig. 7*D*), suggesting that CHOP10 inhibits resistin expression in part by downregulating C/EBPα.

DISCUSSION

The present study demonstrates for the first time that ER stress, which exists in obese adipose tissue, can downregulate resistin in vitro in mouse adipocytes. The effect of ER stress induction appeared to be primarily transcriptional, and three transcription factors were implicated in mediating the effects of ER stress on resistin levels. C/EBPα and PPARγ, which are known activators of resistin, are downregulated by treatment with tunicamycin and have diminished binding at the resistin gene. Knockdown of these factors mimicked the effects of ER stress induction. On the other hand, CHOP10 is a transcriptional repressor that is activated by ER stress in adipocytes and can interact in a dominant negative fashion with C/EBPα (37,38). Knockdown of this protein was able to partially rescue the effects of tunicamycin on resistin expression, suggesting that CHOP10 may also be responsible for the changes in resistin mRNA. Thus, the effects of ER stress on
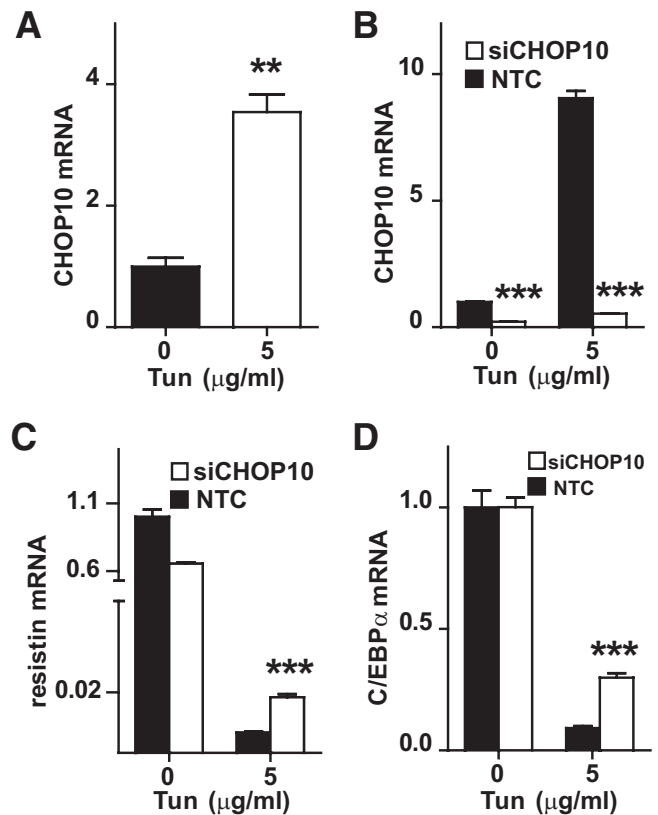
resistin levels in mouse adipocytes appear to be a combination of decreased expression of activating transcription factors, including C/EBPα and PPARγ, and increased expression of repressors such as CHOP10 and possibly others.

A remaining question is how activation of the unfolded protein response leads to downregulation of C/EBPα and PPARγ. One possibility is that repressors of gene transcription such as CHOP10 and ATF3 that are activated as part of the ER stress response may be directly involved. For example, it is known that both C/EBPα and PPARγ genes are activated by C/EBPα binding to their promoters (30,39), and therefore CHOP10 may decrease their expression via its dominant negative interactions with C/EBPα. Notably, however, CHOP10 knockdown was not able to fully rescue the effects of tunicamycin treatment on C/EBPα, indicating that other factors must be involved. Knockdown of ATF3, which is another transcriptional repressor (40) that is activated by ER stress (26), did not affect the ability of tunicamycin to reduce resistin levels (data not shown), suggesting that it does not play a role in this process.

The implication of these findings is that ER stress activation may provide an explanation for the decrease in resistin mRNA in adipose tissue of obese mice in the setting of elevated serum resistin levels. Indeed, this study

demonstrates that decreased resistin expression co-exists with markers of ER stress activation such as increased BiP mRNA and phospho-eIF2α in adipose tissue from obese mice fed an HFD. Furthermore, C/EBPα mRNA and protein were decreased under these conditions, consistent with the role of this transcription factor as an important regulator of resistin (31). In addition, the study demonstrates that in EWAT the levels of resistin protein reflect the downregulation in the mRNA, similar to what is observed for 3T3-L1 adipocytes in vitro. This raises the possibility that the discrepancy between adipose tissue and plasma resistin levels may not be occurring at the level of individual adipocytes but rather results from various global defects characteristic of obesity and insulin resistance. For example, it has been shown that the development of obesity is associated with an increase in fat cell number (41,42), and therefore the net effect in obesity may be elevated resistin release into the circulation even if resistin secretion is decreased on a per-cell basis. In addition, a number of recent studies have demonstrated a negative correlation between renal function and resistin levels (43,44), suggesting that resistin may be cleared through the kidney. Thus, in the setting of diabetic nephropathy, resistin clearance may be impaired leading to accumulation of the protein in the circulation. Another possibility is that resistin half-life in obesity may be increased because of oligomerization. A number of studies have shown that both mouse and human resistin can form oligomers, which can be detected in the circulation (45) and have different biological actions compared with the monomer form (45,46); and the propensity to oligomerize is concentration dependent (47).

It has been previously hypothesized that the discrepancy between resistin mRNA and circulating protein levels may be because of the hyperinsulinemia associated with obesity and insulin resistance (15). In vitro experiments have demonstrated that insulin treatment of mature adipocytes downregulates resistin expression (15,48,49) although the mechanism has not been elucidated. However, there is evidence that insulin can potently decrease C/EBPα expression in differentiated 3T3-L1 cells leading to decreased C/EBPα binding at target DNA sequences (50). This suggests that both ER stress and hyperinsulinemia may contribute to the decreased resistin mRNA levels in obesity by converging on C/EBPα. Moreover, insulin treatment of 3T3-L1 adipocytes did not lead to a discrepancy between resistin mRNA and protein secretion (data not shown), suggesting that at least in this model system both insulin and ER stress downregulated the protein levels along with the mRNA of resistin. It should be noted that the effects of insulin and ER stress in vivo may be different from those observed in vitro in cultured 3T3-L1 cells. However, 3T3-L1 cells, which are derived from immortalized mouse embryonic fibroblasts and can be differentiated into adipocytes with a hormonal cocktail, are generally considered a valid model for adipocyte function. Moreover, transplantation of 3T3-L1 cells into nude mice has been shown to result in formation of adipose tissue that is essentially identical to normal fat (51), suggesting that this cell line is fully capable of reproducing the in vivo adipocyte phenotype.

The overall significance of the findings presented here is that ER stress, which develops in obese adipose tissue, can affect adipocyte function on many levels including dysregulation of adipokine production. It was previously shown that ER stress can impair insulin signaling both in vitro in adipocytes and in vivo in obese XBP$^{+/-}$ mice, which are unable to respond properly to ER stress, and develop dramatically worse adipose tissue insulin resistance compared with wild-type controls (21). Importantly, treatment of obese mice with chemical chaperones that alleviate ER stress improves signaling through the insulin receptor (52), indicating that the effects of ER stress on adipose tissue insulin resistance may be reversible. Thus, targeting ER stress may constitute a feasible strategy for treating obesity and insulin resistance, although it will be critical to understand the various mechanisms by which ER stress affects adipose tissue, including its effects on adipokine expression and function.

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