

## **Endoplasmic Reticulum Stress Regulates Adipocyte Resistin Expression**

Running title: Regulation of Resistin by ER Stress

Martina I. Lefterova, Shannon E. Mullican, Takuya Tomaru, Mohammed Qatanani, Michael Schupp, and Mitchell A. Lazar

Division of Endocrinology, Diabetes, and Metabolism,  
Department of Medicine and Department of Genetics,  
and The Institute for Diabetes, Obesity, and Metabolism  
University of Pennsylvania School of Medicine  
Philadelphia, PA 19104

**Address Correspondence to:**  
Mitchell A. Lazar, M.D., Ph.D.  
e-mail: [lazar@mail.med.upenn.edu](mailto:lazar@mail.med.upenn.edu)

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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. Here 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 ER 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, due to downregulation of C/EBP $\alpha$  and PPAR $\gamma$  transcriptional activators and upregulation of the transcriptional repressor 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 due to various systemic abnormalities associated with obesity and insulin resistance.

The growing obesity epidemic and the co-morbidities 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 which 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. It 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 to 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 AMPK and increased expression of gluconeogenic enzymes (5; 13-16). Additionally, 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). In turn, 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 mouse models of obesity (15; 22; 23). ER stress was recently shown to downregulate adiponectin (24), and we hypothesized that the decrease in resistin mRNA seen in obese adipose tissue may be due to activation of the ER stress response. We show here 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: C/EBP $\alpha$ , PPAR $\gamma$ , and CHOP10. Altering the levels of these

transcription factors mimics or partially rescues the effects of ER stress on resistin expression. Thus, we have uncovered a previously unknown link between activation of the endoplasmic reticulum stress in mouse adipocytes and resistin, which may be of significance *in vivo* in the setting of obesity.

## RESEARCH DESIGN AND METHODS

**Animals.** Six-week old wild-type male C57Bl/6 mice were placed on high fat diet from Research Diets (45kcal% fat) or normal chow (6 kcal% fat) for 30 weeks. At the end of the study, blood samples for serum resistin measurement were collected. Epididymal 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% fetal bovine serum (U.S. Biotechnologies) and 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen)). Two days post-confluence, the cells were induced to differentiate with standard cocktail consisting of Growth Medium with 1 µM dexamethasone, 10 µg/ml bovine insulin, and 0.5 mM 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 post induction of differentiation, when knockdown experiments or treatment with tunicamycin, thapsigargin, or actinomycin D (all from Sigma) were performed.

**Short interfering RNA (siRNA) oligo transfection.** SMART pool siRNA oligos targeting C/EBPα, PPARγ, CHOP10, resistin

(L-051631-00), as well as a non-target negative control were obtained from Dharmacon. Transfection was performed by electroporation with Nucleofector II and Cell Line Nucleofector Kit V (AMAXA). Electroporated cells were re-seeded in growth medium, and harvested or used for treatment at 24 hours or 48 hours 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 1x phosphate buffered saline (PBS), and resuspended in electroporation buffer (solution V, AMAXA). Approximately 1x10<sup>6</sup> cells 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 16hr, the medium was replaced

to fresh culture medium with vehicle or 5  $\mu\text{g/ml}$  tunicamycin. The cells were incubated for 24 additional hours and luciferase activity was measured by luciferase assay kit (Promega). Light units were normalized to  $\beta$ -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 manufacturer's instructions. Samples were diluted appropriately before loading. Serum resistin levels in the mouse diet-induced obesity study were measured using mouse Adipokine LINCoplex Assay (Millipore) according to manufacturer's instructions. Total cell protein was measured using diluted samples on NanoDrop Spectrophotometer (Thermo Scientific).

**RNA isolation and quantitative polymerase chain reaction (QPCR).** RNA was isolated for cells with the RNeasy Mini Kit or from adipose tissue with the RNeasy Lipid kit (both from Qiagen). Reverse transcription of  $\sim 0.5$   $\mu\text{g}$  of RNA was performed with the RT FOR PCR ADVANTAGE KIT (Clontech) following manufacturer's instructions. 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 ATF3, PPAR $\gamma$ , mouse and human C/EBP $\alpha$  expression was done using TaqMan Gene Expression Assays (Applied Biosystems). Primer sequences used for QPCR analysis of CHOP10 and BiP mRNA were obtained from

Rutkowski et al., 2006 (25). The remaining primer sequences are as follows:

F-mus\_resistin:

TCATTTCCTCCTTTTCCTTT

R-mus\_resistin:

TGGGACACAGTGGCATGCT

F-mus\_Arbp/36b4:

CAACCCAGCTCTGGAGAAAC

R-mus\_Arbp/36b4:

CCAACAGCATATCCCGAATC

**Immunoblotting.** Cell protein extracts were generated in cold whole-cell extract buffer (0.15M NaCl, 0.05M Tris, pH 7.4, 0.005M EDTA, 0.5% NP-40) with Complete protease inhibitors (Roche). Immunoblotting following SDS-PAGE was performed using the following antibodies: anti-C/EBP $\alpha$  (sc-61, Santa Cruz), anti-PPAR $\gamma$  (sc-7273, Santa Cruz), anti-HDAC2 (sc-7899, Santa Cruz), and anti-Ran (610341, BD Biosciences), anti-phospho-eIF2 $\alpha$  (Ser51) (119A11, Cell Signaling), total eIF2 $\alpha$  (sc-11386, Santa Cruz).

**Chromatin immunoprecipitation (ChIP).** Cross-linking of adipocyte proteins and chromatin was performed in 1% Formaldehyde (Sigma), followed by quenching in 125mM glycine and two washes with 1x Phosphate Buffered Saline (Invitrogen). Nuclear pellets were obtained following dounce homogenization in nuclear lysis buffer (20mM Hepes, 0.25M sucrose, 3mM MgCl $_2$ , 0.2% NP-40, 3mM beta mercaptoethanol, 0.4mM PMSF, Complete protease Inhibitor tablets). Sonication was carried out with Bioruptor (Diagenode) in ChIP SDS lysis buffer (50 mM Hepes, 1% SDS, 10 mM EDTA). Immunoprecipitations were performed using  $\sim 100$   $\mu\text{g/ml}$  chromatin, and 10  $\mu\text{g}$  of antibody: anti-C/EBP $\alpha$  (sc-61, Santa Cruz), anti-PPAR $\gamma$  (sc-7196, Santa Cruz), or non-specific 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 non-target 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  
           AGCAGCTGGCACCTAACAG  
 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/endoplasmic reticulum  $Ca^{2+}$  pump, and led to dramatically reduced resistin mRNA levels (Fig. 1A). 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. 1B). By contrast, the same treatment induced expression of activating transcription factor 3 (ATF3), which is activated by ER stress (26). The decrease of resistin mRNA by tunicamycin was also dose-dependent (Fig. 1C). In order to examine the effects of tunicamycin on resistin protein levels, cells were treated for 24 h to lower resistin mRNA as in Fig. 1C, 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. 1D), similar to that observed with resistin knockdown, which reduced the mRNA to similar extent but did not induce upregulation of BiP mRNA which would have signified ER stress (Fig. 1E). 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 to 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 vs. lean mice, similar to what is seen in the circulation. To address this question, C57Bl/6 mice were fed a high-fat diet (HFD) or normal chow (NC) for 30 weeks, at which point resistin mRNA levels were decreased in epididymal white adipose tissue (EWAT) of

the HFD mice (Fig. 2A), despite increased serum resistin levels (Fig. 2B). Surprisingly, EWAT resistin protein levels were not increased, but rather tended to be lower in the HFD mice (Fig. 2C). 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-eIF2 $\alpha$  (Fig. 2D) and BiP mRNA levels (Fig. 2E) were elevated in the adipose tissue from the HFD-fed obese mice. Taken together, these data suggest that ER stress may be a relevant mechanism in the downregulation of resistin *in vivo* in the setting of mouse obesity.

**ER 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  $\mu$ g/ml tunicamycin in the presence of 5  $\mu$ g/ml of the transcriptional inhibitor Actinomycin D. Tunicamycin treatment did not reduce the half-life of resistin mRNA as would have been expected if ER stress reduced resistin mRNA by a post-transcriptional mechanism (Fig. 3A). 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  $\mu$ g/ml tunicamycin for 24 hours (Fig. 3B), further demonstrating that ER stress causes reduced resistin gene transcription.

**Downregulation of C/EBP $\alpha$  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 CCAAT/enhancer-binding protein alpha (C/EBP $\alpha$ ). C/EBP $\alpha$  is critical for adipocyte differentiation (28) and contributes to activation of adipocyte-specific genes such as adiponectin (29), PPAR $\alpha$  (30), and resistin (31). A binding site for C/EBP $\alpha$  on the resistin promoter has been characterized and it has been shown that ectopic C/EBP $\alpha$  expression in non-adipogenic cells could drive luciferase expression from the resistin promoter (31). Furthermore, C/EBP $\alpha$  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/EBP $\alpha$  mRNA and protein are reduced in tunicamycin-treated adipocytes (Fig. 4A). This treatment also abolished C/EBP $\alpha$  occupancy at the resistin gene as measured by chromatin immunoprecipitation (ChIP) both at the previously characterized C/EBP $\alpha$  binding site (31) as well as an additional upstream site identified in a recent genome-wide ChIP-chip study (33) (Fig. 4B). Similar decreases in occupancy were noted on the known C/EBP $\alpha$  binding sites on the promoters of PPAR $\gamma$  and adiponectin (data not shown).

Next, we investigated whether knockdown of C/EBP $\alpha$  could recapitulate the effects seen by tunicamycin treatment. For this purpose, mature adipocytes were electroporated with siRNA oligonucleotides against C/EBP $\alpha$  or a non-target control. This strategy reduced C/EBP $\alpha$  protein to similar levels as what is seen in control cells treated with 5  $\mu$ g/ml tunicamycin (Fig. 4C). Resistin mRNA levels, measured 48 hours after electroporation, appeared greatly reduced when C/EBP $\alpha$  had been knocked down (Fig. 4D). Interestingly, measurement of C/EBP $\alpha$  mRNA (Fig. 5A) and protein (Fig. 5B) in WAT of obese mice revealed that C/EBP $\alpha$  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/EBP $\alpha$  levels, which may account for the effects seen on resistin expression. Of note, C/EBP $\beta$  levels were not significantly changed in adipocytes by tunicamycin treatment or by high fat diet feeding in WAT (data not shown).

**Decreased PPAR $\gamma$  expression and activity also contribute to the effects of ER stress.**

Our recent genome-wide study of C/EBP and PPAR $\gamma$  binding in adipocytes (33) demonstrated that PPAR $\gamma$  binds near C/EBP $\alpha$  at the upstream resistin enhancer. PPAR $\gamma$ , which is crucial for adipogenesis (34; 35), also induces resistin expression during adipocyte differentiation (36). Therefore, we examined whether changes in PPAR $\gamma$  levels may also mediate the effects of ER stress on resistin expression. Indeed, tunicamycin treatment of adipocytes substantially reduced PPAR $\gamma$  gene expression (Fig. 6A) as has been reported previously (24). Moreover, tunicamycin treatment nearly abolished recruitment of PPAR $\gamma$  to the resistin gene (Fig. 6B). Furthermore, knockdown of PPAR $\gamma$  in mature adipocytes reduced resistin mRNA levels by approximately 90% (Fig. 6C). Efficiency of PPAR $\gamma$  knockdown was assessed by measuring PPAR $\gamma$  mRNA levels by QPCR (Fig. 6D). Thus, PPAR $\gamma$  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 C/EBP homologous protein 10 (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 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. 7A). Knockdown of CHOP10 (Fig. 7B) partially prevented the tunicamycin-induced decrease in adipocyte resistin mRNA (Fig. 7C). CHOP10 depletion also increased C/EBP $\alpha$  expression in tunicamycin-treated adipocytes (Fig. 7D), suggesting that CHOP10 inhibits resistin expression in part by downregulating C/EBP $\alpha$ .

**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/EBP $\alpha$  and PPAR $\gamma$ , 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/EBP $\alpha$  (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/EBP $\alpha$  and PPAR $\gamma$ , 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/EBP $\alpha$  and PPAR $\gamma$ . 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/EBP $\alpha$  and PPAR $\gamma$  genes are activated by C/EBP $\alpha$  binding to their promoters (30; 39), and therefore CHOP10 may decrease their expression via its dominant negative interactions with C/EBP $\alpha$ . Notably, however, CHOP10 knockdown was not able to fully rescue the effects of tunicamycin treatment on C/EBP $\alpha$ , 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-eIF2 $\alpha$  in adipose tissue from obese mice fed a high-fat diet. Furthermore, C/EBP $\alpha$  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 (43; 44), and therefore the net effect in obesity may be elevated resistin release into the circulation even if resistin secretion is

decreased on per-cell basis. Secondly, a number of recent studies have demonstrated a negative correlation between renal function and resistin levels (41; 42), 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 to 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 due to 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/EBP $\alpha$  expression in differentiated 3T3-L1 cells leading to decreased C/EBP $\alpha$  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/EBP $\alpha$ . 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 downregulate 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<sup>+/-</sup> mice, which are unable to respond properly to ER stress, and develop dramatically worse adipose tissue insulin resistance compared to 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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## FIGURE LEGENDS

**Figure 1. ER stress activation downregulates resistin expression *in vitro* in 3T3-L1 adipocytes.** A. Downregulation of resistin mRNA levels following treatment with 50nM thapsigargin for 24 hours. B. Time course of resistin (rectangles) and Activating transcription factor 3 (ATF3, triangles) mRNA gene expression upon induction of ER stress with 5  $\mu$ g/ml tunicamycin (Tun), presented as fold change over the levels at 0 hours. C. Resistin gene expression in response to vehicle (Veh) and various doses of Tun for 24 hours, presented as fold change over Veh alone. D. Resistin protein concentration in tissue culture media and whole cell lysates presented as ng/ml per mg of total cell protein. Mature adipocytes were electroporated with resistin (siResistin) or non-target control (NTC) siRNA oligos and treated with Veh or 5  $\mu$ g/ml Tun. Twenty-four hours later the cells were washed and treated with Veh or 5  $\mu$ g/ml Tun for 24 hours and resistin protein levels were assayed with ELISA, Mean + SEM, n = 3. E. Gene expression validation that siResistin and Tun treatment reduced resistin mRNA to similar levels, and only Tun induced markers of ER stress such as BiP. Data in (A)-(C) and (E) were normalized to the house-keeping gene Arbp/36b4 and shown as Mean + SEM, n = 3.

**Figure 2. Reduced resistin levels in epididymal white adipose tissue (EWAT) of obese mice are associated with markers of endoplasmic reticulum stress.** All data are from male C57Bl/6 mice fed normal chow (NC, n = 5) or high-fat diet (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 + SEM. 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 ng resistin per mg total protein, Mean + SEM. D. Western blot demonstrating the phosphorylation status of Eukaryotic translation initiation factor 2a (eIF2 $\alpha$ ) in representative animals selected at random from the NC and HFD groups. Total eIF2 $\alpha$  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 presented as Mean + SEM.

**Figure 3. ER stress downregulates resistin in adipocytes by a transcriptional mechanism.** A. Changes in resistin mRNA levels over 48 hours in response to vehicle (Veh, rectangles, solid) or 5  $\mu$ g/ml tunicamycin (Tun, triangles, hatched) in the presence of 5  $\mu$ g/ml Actinomycin D (Act D). Data are presented as per cent of the mRNA level at time 0, Mean  $\pm$  SD of a triplicate experiment. B. Normalized luciferase activity of resistin (-13,580 bp to +243 bp)-luciferase (resistin-luc) or empty vector (pGL3 basic) in the presence of Veh or 5  $\mu$ g/ml Tun for 24 hours. Data are presented as Mean + SEM, n = 3. \*\* p = 0.003 for pGL3 basic vs resistin-luc activity in Veh-treated cells. <sup>##</sup> p = 0.005 for resistin-luc activity in Veh- vs Tun-treated cells.

**Figure 4. Downregulation of C/EBP $\alpha$  by ER stress decreases resistin expression.** A. ER stress induced by treatment with 5  $\mu$ g/ml tunicamycin (Tun) for 24 hours leads to downregulation of C/EBP $\alpha$  mRNA and protein compared to vehicle control (Veh). Gene expression data are presented as Mean + SEM, n = 3. C/EBP $\alpha$  protein levels were assayed by Western Blotting, and HDAC2 served as a loading control. B. ER stress reduces C/EBP $\alpha$  recruitment to resistin at a downstream site (dnstr resistin) at the known C/EBP $\alpha$  binding site at -50 base pairs relative to the transcription start site (TSS), and at a site located approximately 9 kb upstream of the TSS

(upstr resistin). 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/EBP $\alpha$  recruitment. Enrichment was normalized to a site on the Arbp/36b4 gene where C/EBP $\alpha$  does not bind. Data are presented as Mean + SEM of three independent chromatin immunoprecipitation (ChIP) experiments. C. C/EBP $\alpha$  knockdown (siC/EBP $\alpha$ ) mimics the effects of ER stress on resistin expression. Mature adipocytes, electroporated with C/EBP $\alpha$  or non-target control (NTC) siRNA oligos, were treated with vehicle or 5  $\mu$ g/ml tunicamycin (Tun) for 24 hours. D. Efficiency of siC/EBP $\alpha$  was assayed by Western blot and compared to NTC in the presence or absence of 5  $\mu$ g/ml Tun. HDAC2 was used as a loading control. E. Resistin mRNA levels were measured in vehicle-treated treated C/EBP $\alpha$  kd or NTC cells by QPCR and normalized to Arbp/36b4. Data are presented as Mean + SEM, n = 3.

**Figure 5. C/EBP $\alpha$  is downregulated in epididymal white adipose tissue (EWAT) of obese mice.** All data were derived from EWAT of the same animals as in Figure 1; normal chow (NC, n = 5) or high-fat diet (HFD, n = 5). A. C/EBP $\alpha$  mRNA levels normalized to Arbp/36b4, and presented as Mean + SEM. B. C/EBP $\alpha$  protein levels were measured by Western Blotting in EWAT of two randomly selected animals per group. HDAC2 was used as a loading control.

**Figure 6. Downregulation of PPAR $\gamma$  contributes to the effects of ER stress on resistin expression.** A. PPAR $\gamma$  protein levels were measured by Western Blotting in mature adipocytes treated with vehicle (Veh) or 5  $\mu$ g/ml tunicamycin (Tun) for 24 hours. Ran was used as a loading control. B. ER stress decreases recruitment of PPAR $\gamma$  at a binding site near the resistin gene. ChIP-QPCR was performed as in Figure 4B. A region at the insulin gene was used as negative control for PPAR $\gamma$  recruitment. Data are presented as Mean + SEM of three independent chromatin immunoprecipitation (ChIP) experiments. C. PPAR $\gamma$  knockdown (siPPAR $\gamma$ ) can mimic the effects of ER stress on resistin. Mature adipocytes were electroporated with PPAR $\gamma$  (siPPAR $\gamma$ ) or non-target control (NTC) siRNA oligos. Resistin mRNA levels were measured 48 hours later by QPCR and normalized to Arbp/36b4. D. Efficiency of PPAR $\gamma$  knockdown was assayed by QPCR. (C)-(D) Data are presented as Mean + SEM of three replicates.

**Figure 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  $\mu$ g/ml tunicamycin (Tun) for 24 hours. (B)-(D) CHOP10 knockdown (siCHOP10) partially rescues the effects of ER stress on resistin and C/EBP $\alpha$  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  $\mu$ g/ml tunicamycin for 24 hours. D. Effects of siCHOP10 on C/EBP $\alpha$  mRNA levels. All QPCR data were normalized to Arbp/36b4 and presented as Mean + SEM of three replicates.

Figure 1

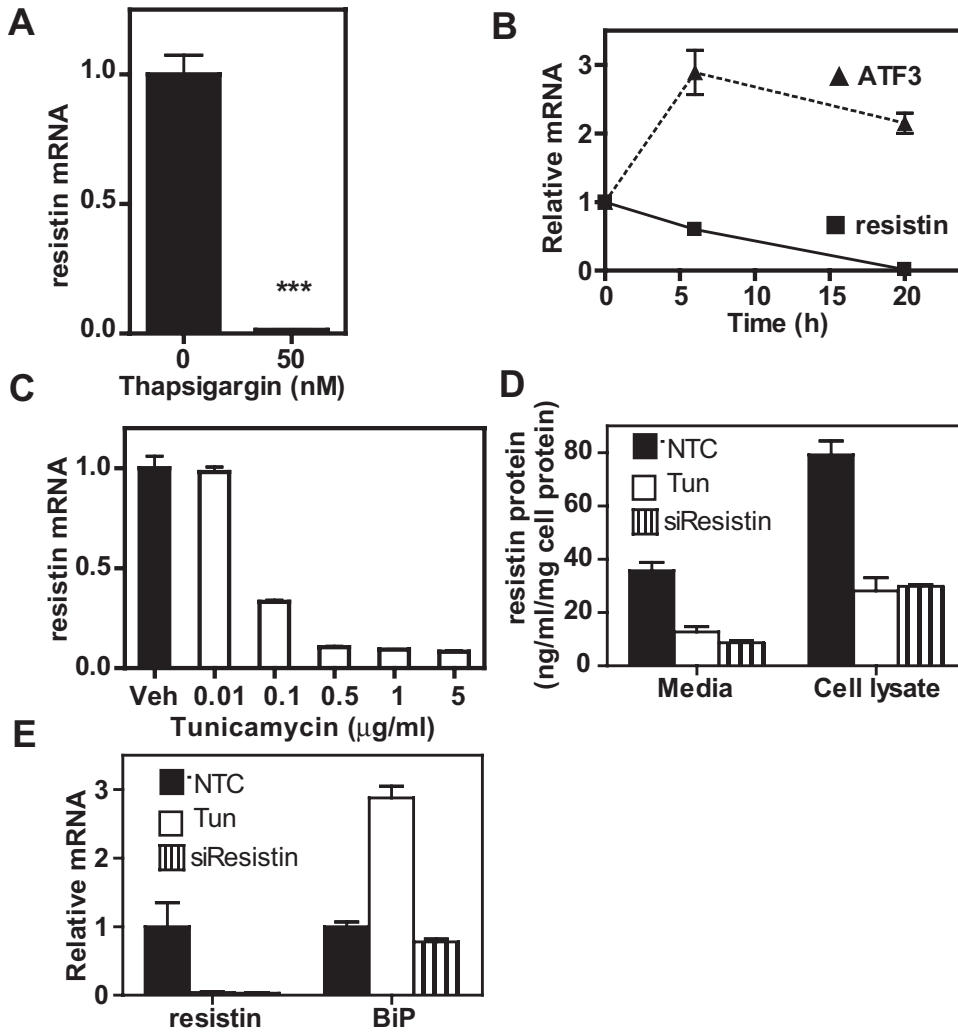


Figure 2

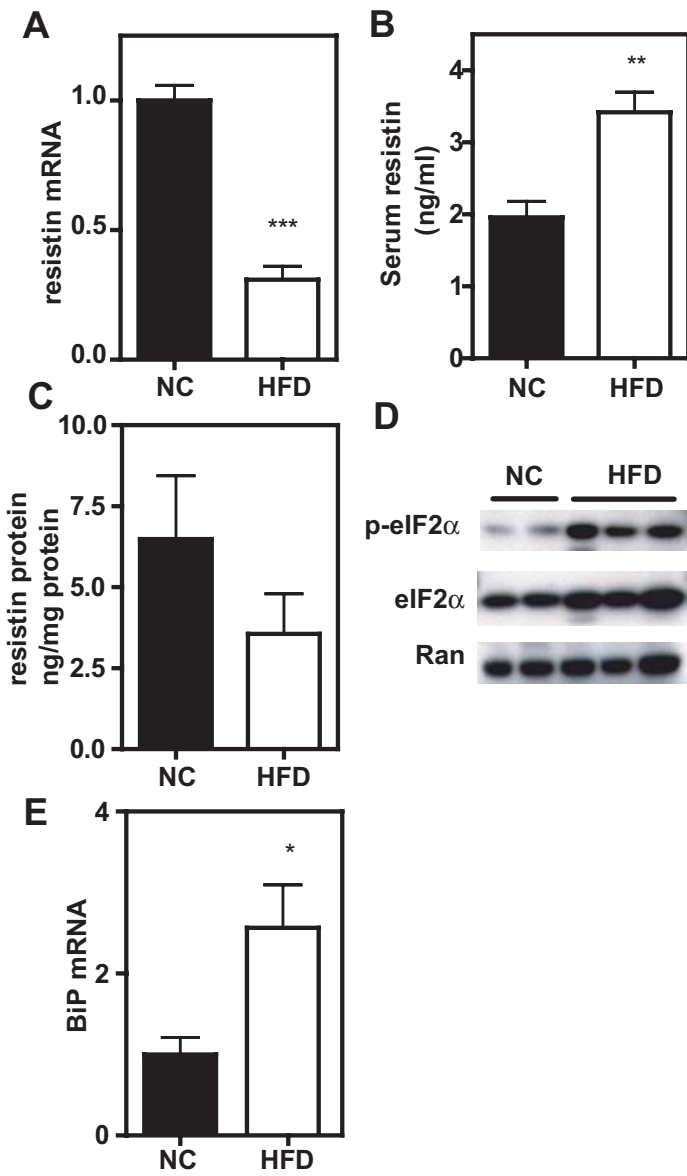


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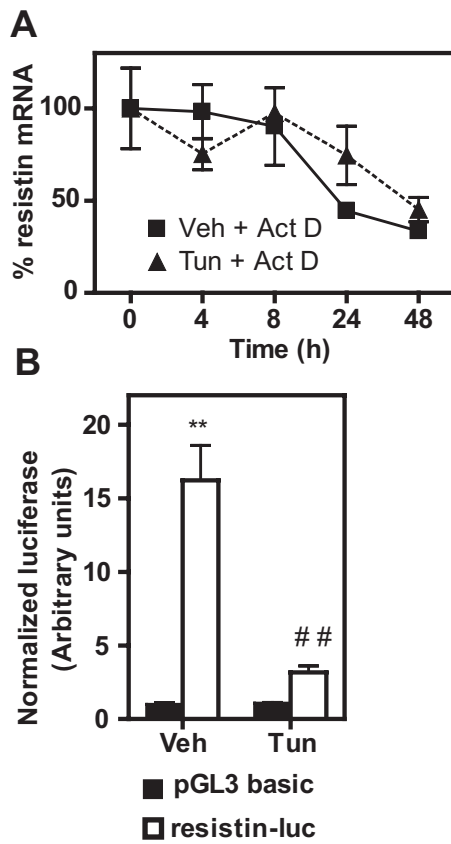


Figure 4

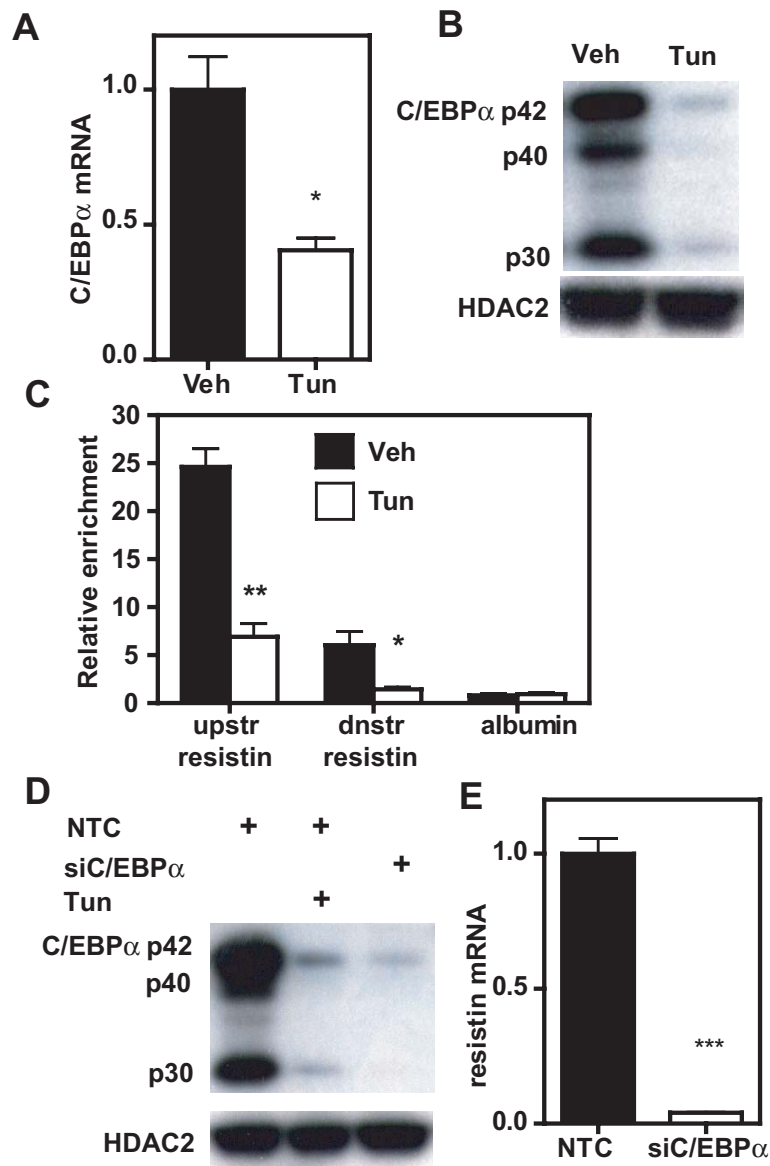


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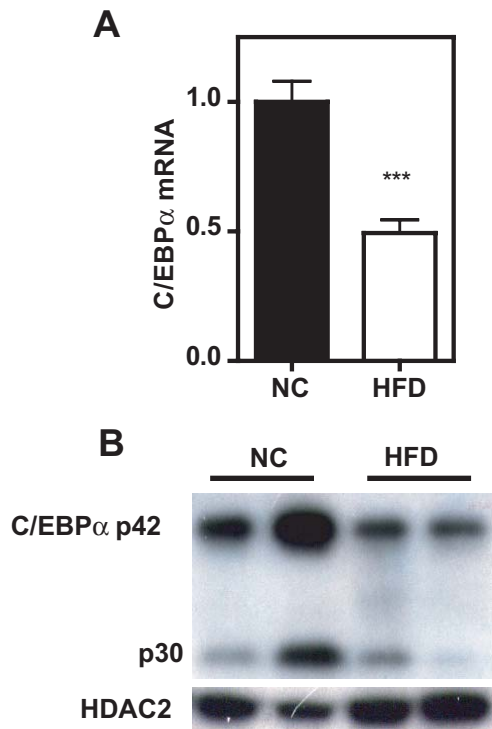


Figure 6

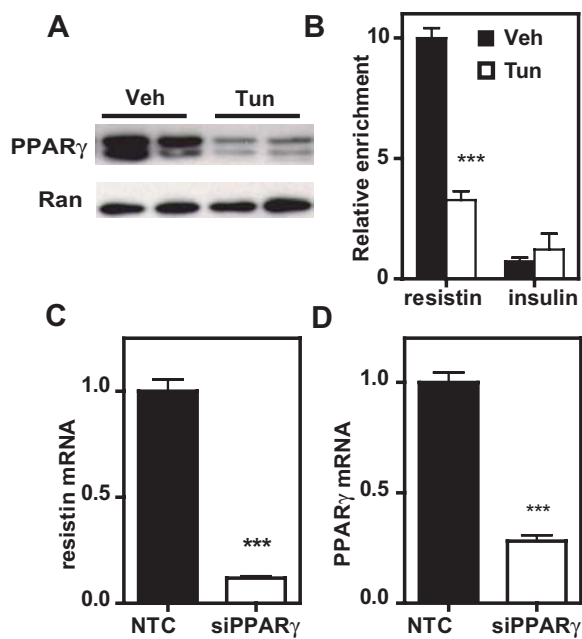


Figure 7

