

Endoplasmic reticulum stress induced by tunicamycin increases resistin messenger ribonucleic acid through the pancreatic endoplasmic reticulum eukaryotic initiation factor 2 α kinase–activating transcription factor 4–CAAT/enhancer binding protein- α homologous protein pathway in THP-1 human monocytes

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Keywords

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ABSTRACT

Aims/Introduction: Resistin, secreted from adipocytes, causes insulin resistance in mice. In humans, the resistin gene is mainly expressed in monocytes and macrophages. Tunicamycin is known to induce endoplasmic reticulum (ER) stress, and reduce resistin gene expression in 3T3-L1 mouse adipocytes. The aim of the present study was to examine whether ER stress affects resistin gene expression in human monocytes.

Materials and Methods: The relationship between resistin messenger ribonucleic acid (mRNA) and ER stress markers mRNA was analyzed by reverse transcription polymerase chain reaction in isolated monocytes of 30 healthy volunteers. The effect of endotoxin/lipopolysaccharides or tunicamycin on resistin gene expression was analyzed in THP-1 human monocytes. Signaling pathways leading to resistin mRNA were assessed by the knockdown using small interfering RNA or overexpression of key molecules involved in unfolded protein response.

Results: Resistin mRNA was positively associated with immunoglobulin heavy chain-binding protein (BiP) or CAAT/enhancer binding protein- α homologous protein (CHOP) mRNA in human isolated monocytes. In THP-1 cells, lipopolysaccharides increased mRNA of BiP, pancreatic endoplasmic reticulum eukaryotic initiation factor 2 α kinase (PERK) and CHOP, as well as resistin. Tunicamycin also increased resistin mRNA. This induction appeared to be dose- and time-dependent. Tunicamycin-induced resistin mRNA was inhibited by chemical chaperone, 4-phenylbutyric acid. The knockdown of either PERK, activating transcription factor 4 (ATF4) or CHOP reduced tunicamycin-induced resistin mRNA. Conversely, overexpression of ATF4 or CHOP increased resistin mRNA.

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Conclusions: Endoplasmic reticulum stress induced by tunicamycin increased resistin mRNA through the PERK–ATF4–CHOP pathway in THP-1 human monocytes. ER stress could lead to insulin resistance through enhanced resistin gene expression in human monocytes.

INTRODUCTION

Resistin is secreted from adipocytes in mice, and its serum levels have been reported to be increased in obese diabetic mice^{1,2}. Treatment with peroxisome proliferator-activated receptor- γ (PPAR- γ) ligands reduces serum resistin in mice. Mice that overexpress the mouse resistin gene (*retn*) in the liver have high serum resistin and are insulin resistant³. *Retn* (–/–) mice show lower insulin resistance and fasting blood glucose⁴. In humans, the human resistin gene (*RETN*) is not expressed in adipocytes, but is highly expressed in monocytes and macrophages⁵, suggesting that its pathophysiological relevance might be different between humans and mice.

We previously carried out a systematic search for single nucleotide polymorphisms (SNPs) in *RETN*, and reported that the G/G genotype of a promoter SNP at –420 was associated with susceptibility to type 2 diabetes mellitus^{6,7}. In addition, we reported that subjects with type 2 diabetes mellitus had higher plasma resistin than subjects without type 2 diabetes mellitus⁸. In the Japanese general population, plasma resistin was highest in the G/G genotype, followed by C/G and C/C⁹.

Recent studies have shown that resistin is related to inflammation. Pro-inflammatory stimuli, such as interleukin-6, tumor necrosis factor- α (TNF- α) and lipopolysaccharides (LPS), induce *RETN* expression in primary human macrophages or humans peripheral blood mononuclear cells (PBMC)^{10,11}. In addition, on tissue injury, pro-inflammatory stimuli increased the expression of monocyte chemoattractant protein (MCP)-1/CCL2 gene, which could trigger endoplasmic reticulum (ER) stress, caused by the accumulation of unfolded or misfolded proteins in the ER lumen^{12–14}. Therefore, resistin gene expression could be influenced by ER stress as well as by inflammation, based on current information.

Eukaryotic cells have an intracellular signaling pathway for reducing ER stress, known as the unfolded protein response^{15,16}. When unfolded or misfolded proteins accumulate, glucose regulated protein 78, also referred to the immunoglobulin heavy chain-binding protein (BiP), releases three critical transmembrane ER signaling proteins, namely, pancreatic ER eukaryotic initiation factor 2 (eIF2) α kinase (PERK), activating transcription factor (ATF) 6, and the inositol requiring enzyme (IRE) 1^{17–19}. PERK phosphorylates the α -subunit of eIF2 α and suppresses protein translation. In contrast, PERK selectively promotes the translation of ATF4, which induces the expression of several genes involved in restoring ER homeostasis. The activation of IRE1 leads to splicing of X-box binding protein 1 (XBP1) messenger ribonucleic acid (mRNA). XBP1 is a transcription factor that induces the expression of several genes

involved in ER-associated degradation. ATF6 induces XBP1 gene transcription, and upregulates the formation of ER molecular chaperons, which, in turn, facilitate protein folding with IRE1^{20,21}. However, when ER stress is prolonged or excessive, unfolded protein response is closely interconnected with pro-inflammatory pathways and/or apoptosis, through the overexpression of CAAT/enhancer binding protein- α (C/EBP) homologous protein (CHOP), the PERK/ATF4-dependent transcription factor. As a result, the cells are unable to maintain ER homeostasis, and finally undergo apoptotic cell death²².

The effect of ER stress on *retn* expression has been studied in 3T3-L1 mouse adipocytes, where the opposite effects were observed. Lefterova *et al.*²³ reported that tunicamycin, an inducer of ER stress, reduced resistin mRNA and protein by suppressing resistin activators, C/EBP and PPAR- γ , in 3T3-L1 adipocytes^{24,25}. In contrast, Chen *et al.*²⁶ reported that oxidized low-density lipoprotein, another ER stress inducer, increased *retn* expression and protein through the upregulation of BiP and CHOP in 3T3-L1 adipocytes²⁷. As the expression of the resistin gene is clearly different between humans and mice, the relationship between ER stress and resistin in human monocytes should be pursued.

In view of this, to elucidate whether ER stress affects *RETN* expression in humans, we examined the relationship between ER stress and *RETN* expression in isolated human monocytes and THP-1 human monocytes. The findings show that ER stress induced by tunicamycin increased resistin mRNA through the PERK–ATF4–CHOP pathway in THP-1 human monocytes.

MATERIALS AND METHODS

Participants

A total of 30 healthy volunteers (11 men and 19 women, age 23.8 ± 2.9 years [range 21–31 years], body mass index 20.3 ± 1.5 [range 17.7–23.3 years]) were analyzed. They had a normal glucose tolerance, as assessed by a 75-g oral glucose tolerance test. Their routine blood tests were within normal ranges. All participants were informed of the purpose of the study and their consent was obtained. The study was approved by the ethics committee of the Ehime University Graduate School of Medicine.

Isolation of human monocytes

Peripheral mononuclear cells were collected from whole blood using a Lymphoprep Tube (Axis-Shield PoC AS, Oslo, Norway), and monocytes were isolated from the mononuclear cells using an anti-CD14 antibody of the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Cell culture and treatment

The human monocytic cell line derived from an acute monocytic leukemia patient, THP-1, was purchased from JCRB (Osaka, Japan) and cultured in Roswell Park Memorial Institute Medium 1640 (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Life Technologies), 2 mmol/L L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. THP-1 cells were grown in a humidified 5% CO₂ atmosphere at 37°C. For experiments with inflammation, THP-1 cells (2×10^5 cells/mL) were treated with 100 ng/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. For experiments with ER stress, THP-1 cells (3×10^5 cells/mL) were treated with 0.01, 0.1, or 1.0 µg/mL tunicamycin (Sigma-Aldrich) for 4, 8, 12, 24, 48 or 72 h. Pharmacological inhibition of ER stress was achieved by preincubating THP-1 cells with the chemical chaperone 4-phenylbutyric acid (4-PBA; Sigma-Aldrich, Steinheim, Germany) at a final concentration of 0.75, 1.5, 3.0 or 7.5 mmol/L for 14 h. After this pretreatment, the cells were treated with 0.1 µg/mL tunicamycin for 24 h.

RNA isolation, reverse transcription and real-time polymerase chain reaction

Total RNA was isolated from THP-1 cells using a RNeasy[®] Mini Kit (QIAGEN, Hilden, Germany). The concentration and purity (A260/A280 ratio) of RNA were determined by NanoDrop 2000[®] (Thermo Fisher Scientific, Waltham, MA, USA). First-strand complementary deoxyribonucleic acid (DNA) was synthesized from 0.5 µg of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription polymerase chain reaction (RT-PCR) was carried out using 12.5 µL of Taqman[®] Universal Master Mix (Applied Biosystems), 1 µL of Taqman[®] Gene Expression Assays (Applied Biosystems) and RNase free water to a final volume of 25 µL. PCR amplification was carried out in an ABI 7500 RT-PCR System (Applied Biosystems) with the cycle conditions (initial cycle at 50°C for 2 min, initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min). Relative RNA levels were calculated by the 2^{-DDCt} method using 18S ribosomal RNA or glyceraldehyde 3-phosphate dehydrogenase as the internal control. The following primers were purchased from Applied Biosystems and used for RT-PCR: glyceraldehyde 3-phosphate dehydrogenase (Hs99999905_m1), 18S (Hs99999901_s1), RETN (Hs00220767_m1), BiP/HSPA5 (Hs00607129_gH), PERK/EIF2AK3 (Hs00984005_m1), ATF4 (Hs00909569_g1), CHOP/DDIT3 (Hs00358796_g1), ATF6α (Hs00232586_m1), IRE1α/ERN1 (Hs00176385_m1) and XBP1 (Hs00231936_m1).

Small interfering RNA transfection

Small interfering RNA (siRNA) oligos targeting PERK, ATF4, CHOP, ATF6, IRE1, XBP1 and a non-target negative control were purchased from Sigma-Aldrich (St. Louis, MO, USA). THP-1 cells were transfected with siRNA using Ingenio[®]

Electroporation Kits and Solution (Mirus Bio Corporation, Madison, WI, USA). The siRNA complexes with 2.5×10^6 THP-1 cells and 500 nmol/L siRNA in 250 µL Ingenio[®] Solution were made and electroporated with a voltage of 250 V and a capacitance of 950 µF using a 0.4-cm cuvette. Thereafter, electroporated cells were added to 50 µL of fresh serum-containing medium in 24-well plates and treated with 0.1 µg/mL tunicamycin for 24 h. The following siRNA were used: negative control (Mission_SIC-001), PERK (Hs_EIF2AK3_6845), ATF4 (Hs_ATF4_2313), CHOP (Hs_DDIT3_3015), ATF6 (Hs_ATF6_3439), IRE1 (Hs_ERN1_4923) and XBP1 (Hs_XBP1_3590).

Overexpression of ATF4 and CHOP

The expression vectors, pCMV6-XL5 containing the coding sequence of human ATF4 (SC119103), and pCMV6-AC containing the coding sequence of human CHOP (SC324377), were purchased from OriGene Technologies, Inc (Rockville, MD, USA). THP-1 cells were transfected with 20 µg of plasmid DNA using Ingenio[®] Electroporation Kits and Solutions. The complexes with 2.5×10^6 THP-1 cells and 20 µg of plasmid in 250 µL Ingenio[®] Solution were made and electroporated with a voltage of 250 V and a capacitance of 950 µF using a 0.4-cm cuvette. Thereafter, electroporated cells were added to 50 µL of fresh serum-containing medium in 24-well plates and allowed to grow for 48 h. It was estimated that at least approximately 17% of the THP-1 cells overexpressed introduced genes by transfection (Figure S1).

Measurements of resistin protein

Cell Lysis

After specific treatments, THP-1 cells frozen with liquid nitrogen were lysed using Cell Lysis Buffer (20 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1 mmol/L Na₂ ethylenediaminetetraacetic acid, 1 mmol/L ethylene glycol tetraacetic acid, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L beta-glycerophosphate, 1 mmol/L Na₃VO₄, 1 µg/mL leupeptin and 1 mmol/L phenylmethylsulfonyl fluoride) with cOmplete Mini protease inhibitor (Roche Applied Science, Mannheim, Germany). The cells were subjected to three cycles of freezing and thawing, and subsequently stirred at 4°C for 1 h. Thereafter, the cells were centrifuged at 14,000 g at 4°C for 10 min. Total cell protein was quantified using the collected supernatant on a Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA).

Human resistin and antibodies for ultrasensitive enzyme-linked immunosorbent assay

Human resistin and mouse anti-human resistin monoclonal antibody (184320; Ab-resistin-1) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Rabbit (anti-2,4-dinitrophenyl [DNP]-BSA) serum and bovine serum albumin (BSA; fraction V) were purchased from Shibayagi Co., Ltd. (Shibukawa, Japan) and InterGen Co. (Purchase, NY, USA),

respectively. Mouse anti-human resistin monoclonal antibody (5G51B22; Ab-resistin-2) was prepared by ourselves. In brief, human resistin complementary DNA (CCDS 12182.1) was amplified using PCR from human leukocytes RNA and subcloned into the pEU-GST(TEV)-N2 vector²⁸. Glutathione S-transferase (GST)-tagged recombinant human resistin protein was automatically synthesized by the Robotic Protein Synthesizer Proteomist[®] DT II (CellFree Sciences, Matsuyama, Japan) according to a cell-free protein synthesis system using wheat germ ribosomal RNA^{29,30}. Monoclonal antibodies were raised against the GST-tagged resistin based on the mouse spleen method. Further information for the preparation of the monoclonal antibody is available on request.

Ultrasensitive enzyme-linked immunosorbent assay, immune complex transfer enzyme immunoassay, for human resistin

Immune complex transfer enzyme immunoassay for human resistin was carried out by following the established method previously described^{31,32}. Precisely, an aliquot (100 μ L) of standard human resistin or samples diluted in buffer A (10 mmol/L sodium phosphate buffer (pH 7.0), containing 0.4 mol/L NaCl, 0.1 g/L BSA, 1 mmol/L MgCl₂ and 1.0 g/L NaN₃) was incubated overnight at 4°C with 100 μ L buffer A containing 30 fmol of Ab-resistin-2 Fab'- β -D-galactosidase, and 10 fmol Ab-resistin-1 immunoglobulin G2b conjugated with DNP and biotin (formation). Thereafter, one polystyrene bead coated with rabbit anti-DNP-BSA immunoglobulin G was added to the mixture and incubated for 30 min (entrapment). After removing the incubation mixture, the polystyrene bead was washed twice with buffer B (10 mmol/L sodium phosphate buffer [pH 7.0], containing 0.1 mol/L NaCl, 1.0 g/L BSA, 1.0 mmol/L MgCl₂ and 1.0 g/L NaN₃) and then incubated in 150 μ L buffer B containing 2 mmol/L *eN*-2,4-DNP-L-lysine for 30 min (elution). After removal of the polystyrene bead, one streptavidin-coated polystyrene bead was added to the eluate and incubated for 30 min (transfer). The bead was then washed, and the bound β -D-galactosidase activity was assayed fluorometrically with 4-methylumbelliferyl- β -D-galactoside (0.2 mmol/L) as a substrate for 20 h at 30°C. The incubations with polystyrene beads were carried out with shaking at 210 strokes/min at room temperature throughout. The within-assay and between-assay coefficient variations (CVs) for resistin were 5.2–9.7% ($n = 5$) and 5.0–9.7% ($n = 5$), respectively. The detection limit and sensitivity for resistin were 0.003 pg/mL and 0.15 pg/mL of medium sample, respectively. The detection limit was 1000-fold less than that of conventional enzyme-linked immunosorbent assay. A typical calibration curve of immune complex transfer enzyme immunoassay for resistin is shown in Figure S2.

Statistical analysis

ANOVA with Scheffe's test or unpaired Student's *t*-test was used for statistical analyses. Simple regression analyses were used for correlation between resistin mRNA and ER stress markers

mRNA in human monocytes. These analyses were carried out using Excel 2010 (Microsoft, Seattle, WA, USA) with the add-in software Statcel 3³³. Differences between groups were considered to be statistically significant at values of $P < 0.05$. All results are reported as the mean \pm the standard error of the mean (SEM).

RESULTS

Resistin mRNA was positively correlated with BiP or CHOP mRNA in human isolated monocytes

To examine the relationship between *RETN* expression and ER stress in human monocytes, we first analyzed the mRNA of resistin and ER stress marker genes, BiP and CHOP, in isolated monocytes obtained from the 30 healthy volunteers. Resistin mRNA was positively correlated with either BiP or CHOP mRNA ($R = 0.453$, $P < 0.05$, and $R = 0.420$, $P < 0.05$, respectively) (Figure 1a,b). Therefore, resistin mRNA could be induced by ER stress in human monocytes.

LPS increased resistin, BiP, PERK and CHOP mRNA in THP-1 human monocytes

We next examined whether LPS affected ER stress markers as well as resistin mRNA in THP-1 human monocytes. Resistin mRNA was increased to ~ 1.5 -fold in the presence of 100 ng/mL of LPS within 24 h (Figure 2). LPS also enhanced mRNA of the ER stress markers, BiP, PERK and CHOP. Therefore, LPS increased resistin and ER stress markers mRNA in THP-1 human monocytes under the same conditions.

ER Stress, induced by tunicamycin, increased resistin mRNA in THP-1 human monocytes

To determine whether ER stress affected resistin mRNA, we examined the effect of tunicamycin, which induces ER stress by inhibiting *N*-linked glycosylation, in THP-1 human monocytes. Tunicamycin treatment increased resistin mRNA for 24 h (Figure 3a). This induction appeared to be dose-dependent, and reached a maximum for 0.1 μ g/mL of tunicamycin. We then examined the time-course for this using a concentration of tunicamycin of 0.1 μ g/mL. The induction of resistin mRNA by tunicamycin appeared to be time-dependent up to 24 h, and then declined to 48 h (Figure 3b). The mRNA of ER stress markers, except for ATF6, was also enhanced by tunicamycin for 24 h (Figure 3c). Therefore, tunicamycin, which induces ER stress, increased resistin mRNA in THP-1 human monocytes. Similarly, thapsigargin, another ER stress inducer, increased resistin mRNA in THP-1 human monocytes (Figure S3a).

4-phenylbutyrate, a chemical chaperone, reduced tunicamycin-induced resistin and ER stress markers mRNA in THP-1 human monocytes

We examined the effect of 4-phenylbutyrate (4-PBA), a chemical chaperone of ER stress, on resistin and ER stress markers mRNA. The 4-PBA inhibited the tunicamycin-induced mRNA

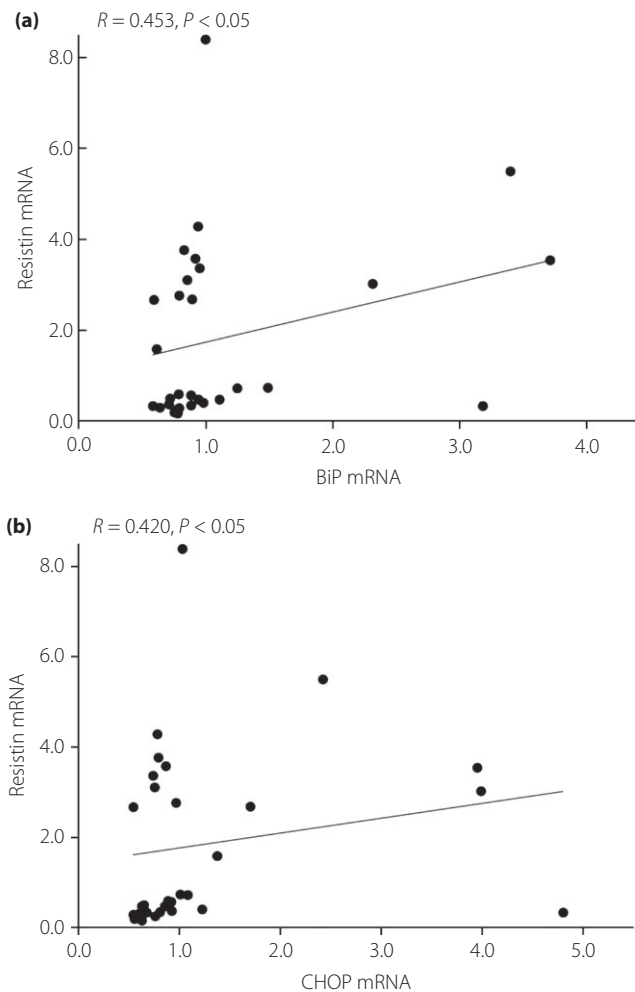


Figure 1 | Resistin messenger ribonucleic acid (mRNA) was positively correlated with immunoglobulin heavy chain-binding protein (BiP) or CAAT/enhancer binding protein- α homologous protein (CHOP) mRNA in human isolated monocytes. The mRNA of resistin, BiP and CHOP in isolated monocytes of 30 healthy volunteers were quantitated using reverse transcription polymerase chain reaction as described in Materials and Methods. The mRNA was corrected by human glyceraldehyde 3-phosphate dehydrogenase mRNA in each sample for meaningful comparison. The relative mRNA of resistin, BiP and CHOP was determined by calculating from the threshold cycles. Simple regression analyses were used for statistical analyses. (a) BiP ($R = 0.453$, $P < 0.05$). (b) CHOP ($R = 0.420$, $P < 0.05$).

of the ER stress markers, BiP, PERK, ATF4, CHOP, IRE1 and XBP1 (Figure 4a). Resistin mRNA induced by tunicamycin was also reduced by 4-PBA (Figure 4b). These effects of 4-PBA appeared to be dose-dependent. The mRNA of resistin and ER stress markers in THP-1 cells without tunicamycin treatment was not affected by 4-PBA (data not shown). Therefore, the 4-PBA reduced tunicamycin-induced resistin and ER stress markers mRNA in THP-1 human monocytes.

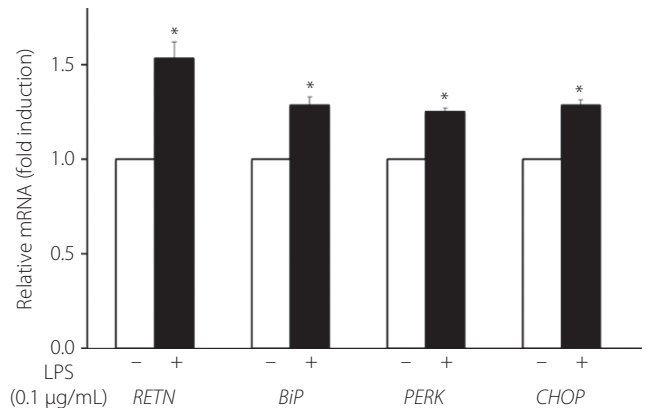


Figure 2 | Lipopolysaccharides (LPS) increased resistin, immunoglobulin heavy chain-binding protein (BiP), pancreatic endoplasmic reticulum eukaryotic initiation factor 2 α kinase (PERK) and CAAT/enhancer binding protein- α homologous protein (CHOP) messenger ribonucleic acid (mRNA) in THP-1 human monocytes. THP-1 human monocytes were treated with 100 ng/mL LPS for 24 h. The mRNA of human resistin and endoplasmic reticulum stress markers, BiP, PERK and CHOP was quantitated using reverse transcription polymerase chain reaction as described in the Materials and Methods. Each mRNA is shown relative to 18S rRNA mRNA. Each mRNA of the control was defined as 1.0. The mean \pm standard error of the mean of six separate experiments with triplicate samples is shown. * $P < 0.0005$ vs control (unpaired Student's *t*-test). *RETN*, human resistin gene.

Knockdown of PERK, ATF4 or CHOP inhibited tunicamycin-induced resistin mRNA in THP-1 human monocytes

To determine the signaling pathway responsible for tunicamycin-induced resistin mRNA, siRNA of key molecules involved in ER stress was used. The siRNA of PERK inhibited tunicamycin-induced resistin mRNA in THP-1 human monocytes (Figure 5a). The siRNA of downstream molecules, ATF4 or CHOP, also reduced tunicamycin-induced resistin mRNA (Figure 5b,c). The siRNA of PERK, ATF4 or CHOP blocked its own mRNA. Resistin mRNA was not affected by the siRNA of ATF6, IRE1 or XBP1, whereas their own mRNA was reduced (Figure S4). Therefore, the PERK–ATF4–CHOP pathway appears to be involved in tunicamycin-induced resistin mRNA in THP-1 monocytes.

Overexpression of ATF4 or CHOP increased resistin mRNA in THP-1 human monocytes

To confirm the involvement of ATF4 and CHOP in the induction of resistin mRNA, the effect of the transient overexpression of ATF4 or CHOP was examined. ATF4 and CHOP were overexpressed (Figure 6a,b). ATF4 increased resistin mRNA by 1.52-fold compared with controls (Figure 6a). Likewise, CHOP induced resistin mRNA by 1.25-fold (Figure 6b). Therefore, ATF4 and CHOP, downstream molecules of PERK in the ER stress signaling pathway, were involved in the tunicamycin-induced resistin mRNA in THP-1 human monocytes.

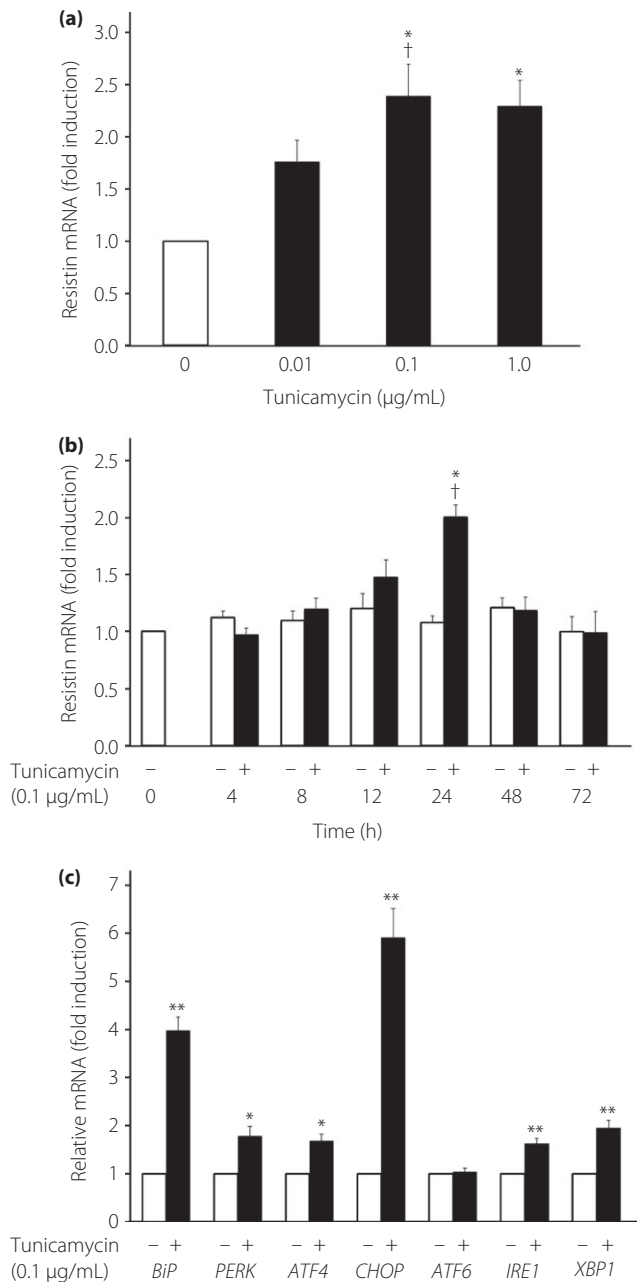
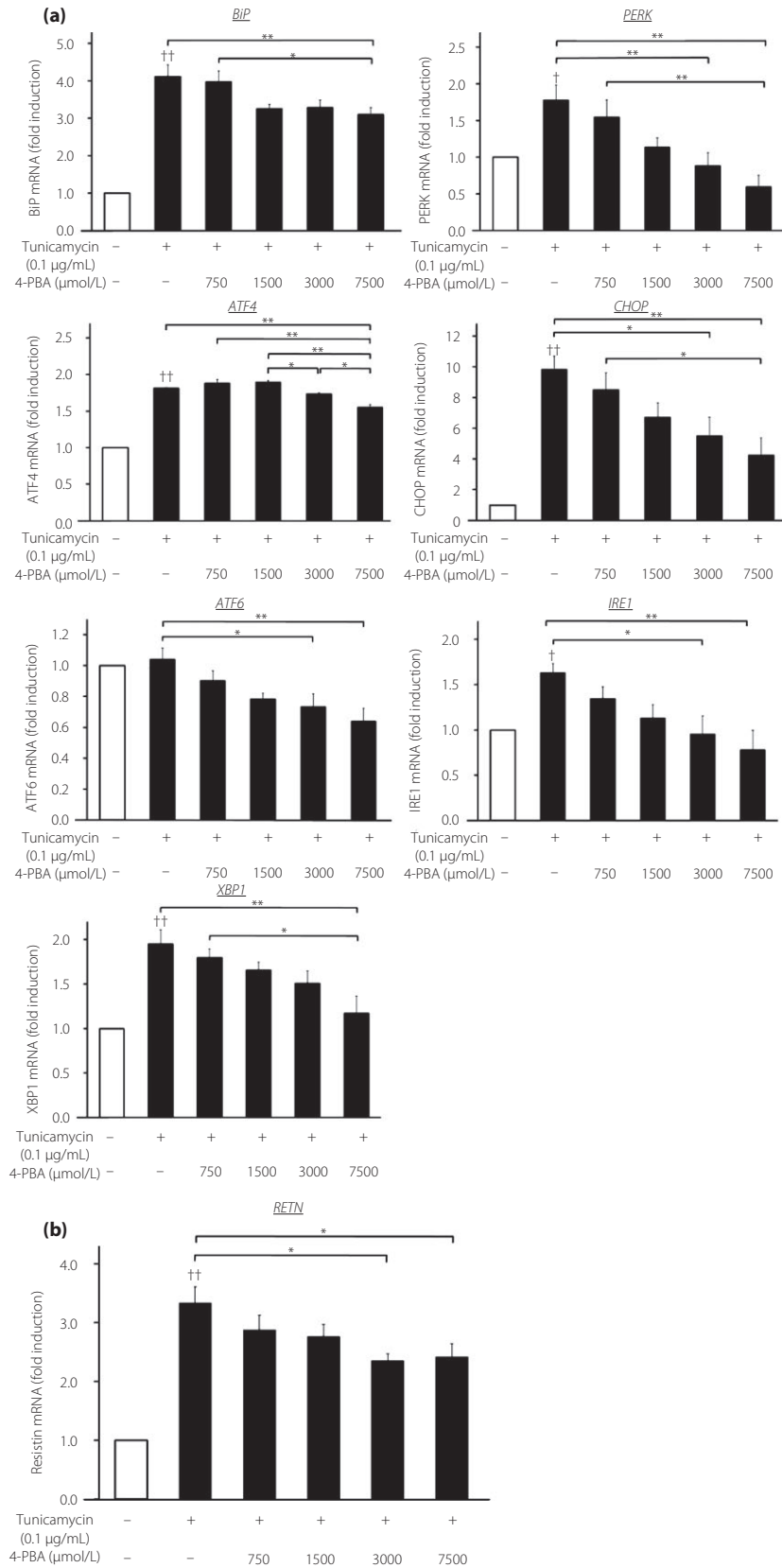


Figure 3 | Endoplasmic reticulum stress, induced by tunicamycin, increased resistin messenger ribonucleic acid (mRNA) in THP-1 human monocytes. (a) Tunicamycin increased resistin mRNA in THP-1 human monocytes (dose–response). THP-1 cells were treated with 0 (control), 0.01, 0.1 or 1.0 µg/mL tunicamycin for 24 h. The mRNA of human resistin was quantitated using reverse transcription polymerase chain reaction, and the data are shown relative to 18S rRNA mRNA as described in the Materials and Methods. The mRNA of the control was defined as 1.0. The mean ± standard error of the mean of six separate experiments with triplicate samples is shown. ANOVA; $P = 0.0014$, Scheffe’s test; $*P < 0.01$ compared with control, $\dagger P < 0.05$ compared with 0.01 µg/mL tunicamycin. (b) (Time-course) THP-1 cells were treated with 0.1 µg/mL tunicamycin for 4, 8, 12, 24, 48 or 72 h. The mRNA of the control (0 h) was defined as 1.0. The mean ± standard error of the mean of six separate experiments with triplicate samples are shown. ANOVA; $P = 2.63 \times 10^{-6}$; Scheffe’s test; $*P < 0.01$ compared with 4 and 72 h in THP-1 cells treated with 0.1 µg/mL tunicamycin, $\dagger P < 0.05$ compared with 8, 12 and 48 h in THP-1 cells treated with 0.1 µg/mL tunicamycin. (c) The mRNA of endoplasmic reticulum stress markers, immunoglobulin heavy chain-binding protein (BiP), pancreatic endoplasmic reticulum eukaryotic initiation factor 2 α kinase (PERK), activating transcription factor 4 (ATF4), CAAT/enhancer binding protein- α homologous protein (CHOP), inositol requiring enzyme 1 (IRE1) or X-box binding protein 1 (XBP1) was increased by tunicamycin. THP-1 cells were treated with 0.1 µg/mL tunicamycin for 24 h. The mRNA of control (tunicamycin [-], 24 h) was defined as 1.0. The mean ± standard error of the mean of six separate experiments with triplicate samples is shown. Unpaired Student’s *t*-test was used for statistical analyses. $*P < 0.01$, $**P < 0.001$ vs control.

Intracellular resistin protein appeared to be enhanced by tunicamycin in THP-1 human monocytes

We next measured resistin protein in the cell lysate of THP-1 cells treated with or without 0.1 µg/mL tunicamycin for 6, 12 or 24 h. Resistin protein in the cell lysate treated without tunicamycin was lower at 24 h than 0 h (Figure 7). In contrast, resistin protein in the cell lysate treated with tunicamycin was maintained at 24 h. When the fold induction of resistin protein by tunicamycin was compared at each time-point, this induction was higher at 24 h than 0 and 6 h. Therefore, intracellular resistin protein appeared to be enhanced under tunicamycin-induced ER stress in THP-1 human monocytes at at least 24 h.

Figure 4 | The 4-phenylbutyrate (4-PBA) reduced tunicamycin-induced resistin and ER stress markers messenger ribonucleic acid (mRNA) in THP-1 human monocytes. THP-1 cells were pre-incubated with 750, 1,500, 3,000 or 7,500 µmol/L of 4-PBA, a chemical chaperone, for 14 h, and 0.1 µg/mL tunicamycin was subsequently added to the medium for 24 h. The mRNA of (a) endoplasmic reticulum stress markers (immunoglobulin heavy chain-binding protein [BiP], pancreatic endoplasmic reticulum eukaryotic initiation factor 2 α kinase [PERK], activating transcription factor 4 [ATF4], CAAT/enhancer binding protein- α homologous protein [CHOP], ATF6, inositol requiring enzyme 1 [IRE1] and X-box binding protein 1 [XBP1]) and (b) resistin (*RETN*) were quantitated using reverse transcription polymerase chain reaction and shown relative to 18S rRNA mRNA as described in the Materials and Methods. The mRNA of control (4-PBA [-], tunicamycin [-]) was defined as 1.0. The mean ± standard error of the mean of five separate experiments with triplicate samples is shown. ANOVA; $P = 8.49 \times 10^{-8}$ (BiP), $P = 0.00040$ (PERK), $P = 2.46 \times 10^{-6}$ (ATF4), $P = 1.53 \times 10^{-5}$ (CHOP), $P = 0.00085$ (ATF6), $P = 0.0070$ (IRE1), $P = 0.00016$ (XBP1), $P = 5.44 \times 10^{-6}$ (RETN); Scheffe’s test; $\dagger\dagger P < 0.01$, $\dagger P < 0.05$ vs control (tunicamycin [-], 4-PBA [-]), $**P < 0.01$, $*P < 0.05$.



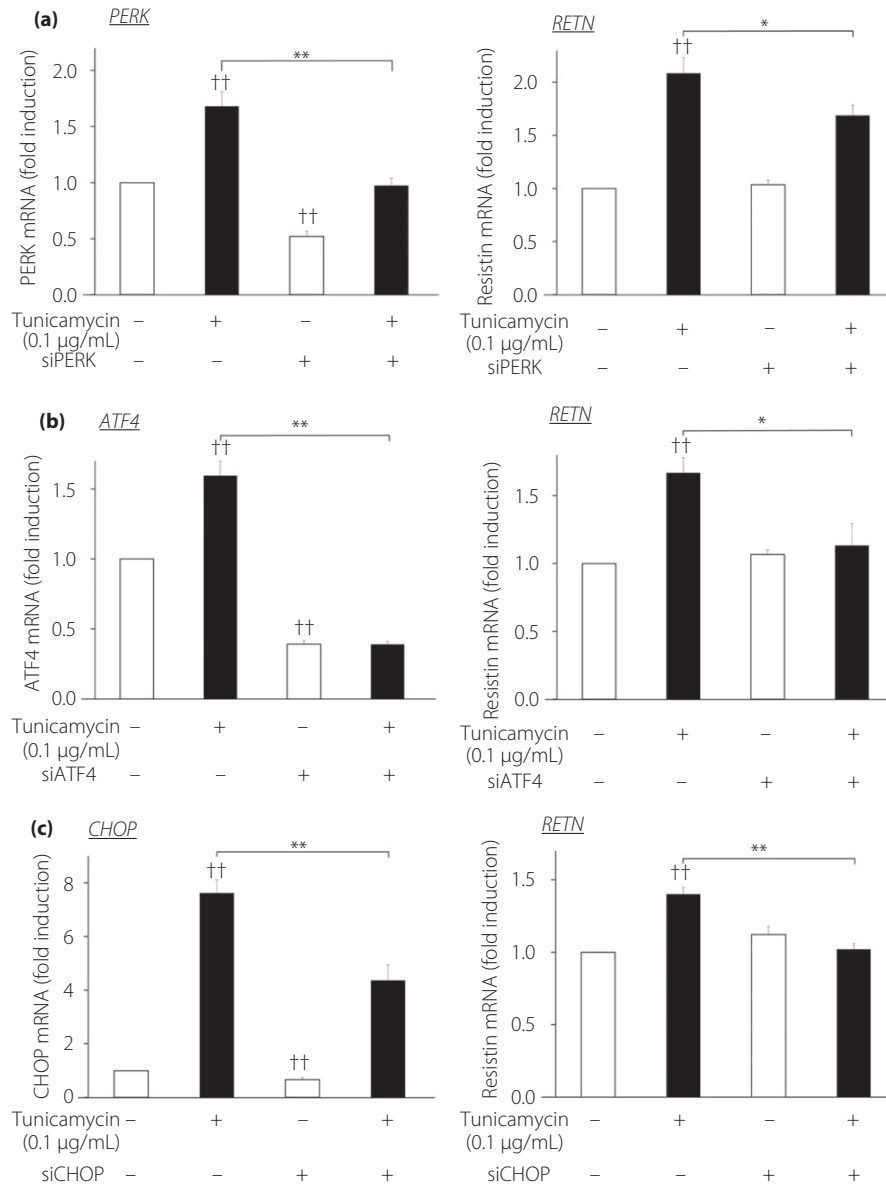


Figure 5 | The knockdown of pancreatic endoplasmic reticulum eukaryotic initiation factor 2 α kinase (PERK), activating transcription factor 4 (ATF4) or CAAT/enhancer binding protein- α homologous protein (CHOP) inhibited tunicamycin-induced resistin messenger ribonucleic acid (mRNA) in THP-1 human monocytes. THP-1 cells were transfected with each of 500 nmol/L small interfering RNA (siRNA), and 0.1 μ g/mL tunicamycin was subsequently added to the medium for 24 h. The mRNA of PERK, ATF4, CHOP and resistin (RETN) was quantitated using reverse transcription polymerase chain reaction, and are shown relative to 18S rRNA mRNA as described in the Materials and Methods. The effect of siRNA for (a) PERK, (b) ATF4 and (c) CHOP. The mRNA of control (tunicamycin [-]) is defined as 1.0. The mean \pm standard error of the mean of six separate experiments with duplicate samples is shown. ANOVA; (a) $P = 1.29 \times 10^{-8}$ (PERK), $P = 3.79 \times 10^{-6}$ (RETN), (b) $P = 4.01 \times 10^{-9}$ (ATF4), $P = 0.0016$ (RETN), (c) $P = 5.85 \times 10^{-11}$ (CHOP), $P = 2.14 \times 10^{-6}$ (RETN), Scheffe's test; †† $P < 0.01$ vs control (tunicamycin [-], siRNA [-]), ** $P < 0.01$, * $P < 0.05$.

Tunicamycin decreased resistin protein secretion from THP-1 human monocytes

Finally, we measured the amount of resistin protein secreted from THP-1 cells into the medium. THP-1 cells were treated with or without 0.1 μ g/mL tunicamycin for 6, 12 or 24 h.

Paradoxically, the resistin protein in the medium was lower with tunicamycin treatment at 12 and 24 h (Figure 8). Therefore, tunicamycin decreased resistin protein secreted from THP-1 human monocytes, while increasing resistin mRNA, and possibly protein in these cells.

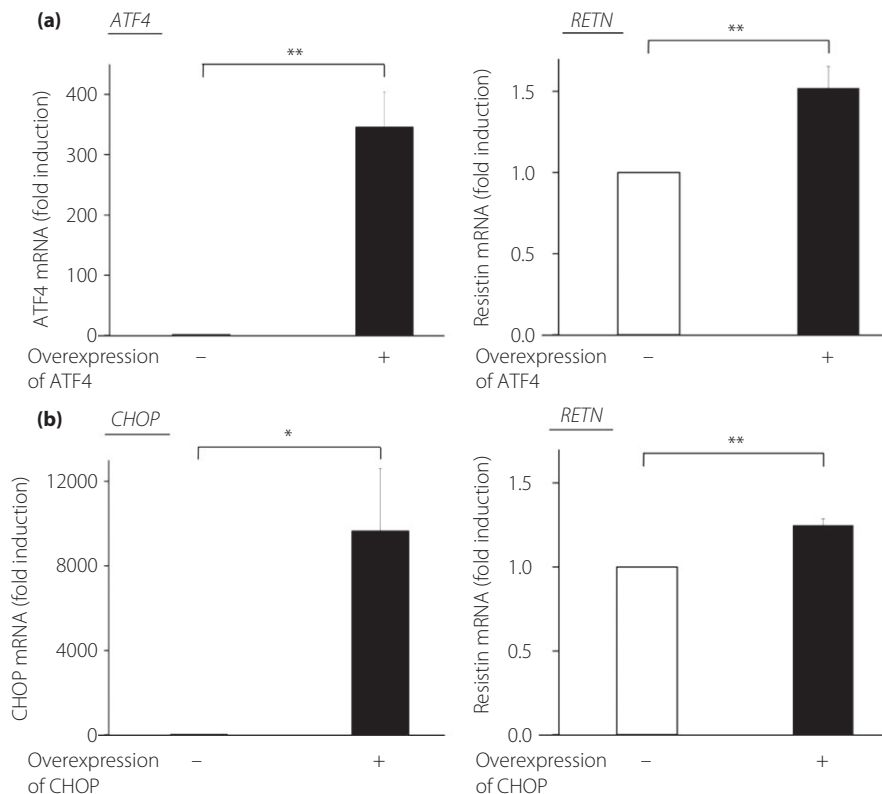


Figure 6 | Overexpression of activating transcription factor 4 (ATF4) or CAAT/enhancer binding protein- α homologous protein (CHOP) increased resistin messenger ribonucleic acid (mRNA) in THP-1 human monocytes. THP-1 cells were transfected with each of 20 μ g of plasmid deoxyribonucleic acid, pCMV6-XL5 containing the coding sequence of human ATF4 or pCMV6-AC containing the coding sequence of human CHOP, and were subsequently incubated in the medium for 48 h. The mRNA of ATF4, CHOP and resistin (*RETN*) was quantitated using reverse transcription polymerase chain reaction and shown relative to 18S rRNA mRNA as described in the Materials and Methods. The mRNA of the control (empty pCMV6-XL5 or pCMV6-AC) is defined as 1.0. The mean \pm standard error of the mean of five separate experiments with duplicate samples is shown. Unpaired Student's *t*-test was used for statistical analyses. ***P* < 0.01, **P* < 0.05.

DISCUSSION

We have shown that ER stress markers mRNA was positively associated with resistin mRNA in isolated human monocytes. ER stress induced by tunicamycin also increased resistin mRNA in THP-1 human monocytes. The PERK-ATF4-CHOP pathway was involved in this increase, as evidenced by knockdown using siRNA and the overexpression of key molecules associated with ER stress.

In the present study, resistin mRNA was positively associated with mRNA of ER stress markers, BiP and CHOP, in isolated monocytes from healthy volunteers. We previously reported that plasma resistin was positively correlated with insulin resistance in the Japanese general population⁹. In addition, Degaspero *et al.*³⁴ reported that activated ER stress was observed in monocytes from obese patients compared with healthy controls. Most recently, Gregor *et al.*³⁵ reported that mRNA of the ER stress markers, BiP and spliced XBP-1, was decreased in subcutaneous abdominal adipose tissues in obese subjects, when insulin resistance was improved by weight loss after gastric bypass

surgery. Therefore, ER stress appears to be positively associated with resistin mRNA in human monocytes, as well as insulin resistance.

We found that LPS, a typical inducer of *RETN* expression, also increased mRNA of BiP, PERK and CHOP in THP-1 human monocytes. Lehrke *et al.*¹¹ reported that LPS increased *RETN* expression in primary human macrophages, which was inhibited by anti-inflammatory drugs or the PPAR- γ ligand. Therefore, inflammatory stimuli, such as LPS, could induce resistin mRNA through ER stress in human monocytes.

We found that ER stress induced by tunicamycin increased resistin mRNA in THP-1 human monocytes. This induction appeared to be time-dependent for periods of up to 24 h, and then declined to 48 h. Cell death caused by ER stress-induced apoptosis could account for this decline. In contrast, previous studies have reported that the expression of *retn* was downregulated by LPS or tunicamycin-induced ER stress in 3T3-L1 mouse adipocytes^{23,36}. The opposite effects of LPS or tunicamycin between human monocytes and mouse adipocytes sug-

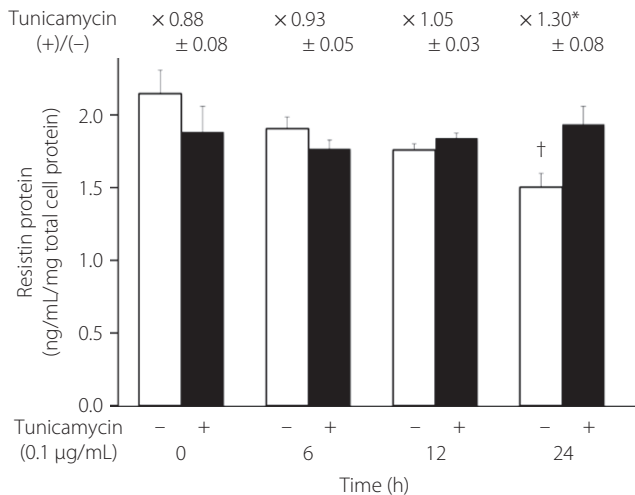


Figure 7 | Intracellular resistin protein appeared to be enhanced by tunicamycin in THP-1 human monocytes. THP-1 cells were treated with 0.1 $\mu\text{g/mL}$ tunicamycin for 6, 12 or 24 h. The resistin protein in the cell lysate was measured using immune complex transfer enzyme immunoassay (ICT-EIA) as described in the Materials and Methods. The mean \pm standard error of the mean of six separate experiments with triplicate samples is shown. ANOVA and Scheffe's test were used for statistical analyses. ANOVA; $P = 0.021$, Scheffe's test; † $P < 0.01$ vs control (0 h, tunicamycin [-]). In addition, the ratio of resistin protein in the cell lysate treated with tunicamycin to that untreated at each time-point was shown in the upper part of the panel (mean \pm standard error of the mean). ANOVA; $P = 0.0014$, Scheffe's test; * $P < 0.01$ vs 0 and 6 h.

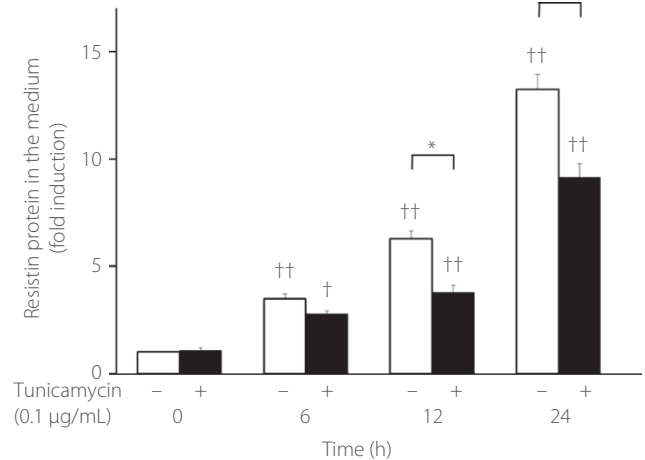


Figure 8 | Tunicamycin decreased resistin protein secretion from THP-1 human monocytes. THP-1 cells were treated with 0.1 $\mu\text{g/mL}$ tunicamycin for 6, 12 or 24 h. The resistin protein in the medium was measured using immune complex transfer enzyme immunoassay (ICT-EIA) as described in the Materials and Methods. The resistin protein of the control (0 h, tunicamycin [-]) was defined as 1.0. The mean \pm standard error of the mean of six separate experiments with triplicate samples is shown. ANOVA and Scheffe's test were used for statistical analyses. ANOVA; $P = 3.40 \times 10^{-25}$, Scheffe's test; †† $P < 0.01$, † $P < 0.05$ vs control (0 h, tunicamycin [-]), * $P < 0.01$ (tunicamycin [+]) vs tunicamycin [-] at 12 and 24 h.

gest that resistin gene expression was differentially regulated between these cells.

We showed that 4-PBA reduced tunicamycin-induced resistin mRNA in THP-1 cells. The 4-PBA also inhibited tunicamycin-induced mRNA of typical molecules involved in ER stress in THP-1 cells. The 4-PBA is known to be a representative chemical chaperone of ER stress, and functions to reduce the ER stress-induced phosphorylation of PERK and IRE-1, and the splicing of XBP1 mRNA in the liver of *ob/ob* mice^{37,38}. Kawasaki *et al.*³⁹ reported that 4-PBA inhibited the gene expression of inflammatory cytokines induced by a high-fat diet through ER stress in mice. Zeng *et al.*⁴⁰ reported that 4-PBA suppressed the migration of THP-1 monocytes induced by ER stress-mediated inflammation by inhibiting BiP and PERK gene expression. Therefore, 4-PBA could reduce tunicamycin-induced *RETN* expression by inhibiting ER stress in THP-1 cells.

We found that the siRNA of either PERK, ATF4 or CHOP inhibited tunicamycin-induced resistin mRNA in THP-1 human monocytes. The siRNA of ATF6, IRE1 or XBP1 had no effect on the tunicamycin-induced resistin mRNA. Conversely, the overexpression of ATF4 and CHOP increased resistin mRNA. In addition, the induction of CHOP mRNA by tunicamycin appeared to be time-dependent up to 8 h, and then declined to 24 h (data not shown), whereas that of resistin

mRNA reached a peak later at 24 h. Therefore, the PERK-ATF4-CHOP pathway appears to be mainly involved in tunicamycin-induced resistin mRNA in THP-1 human monocytes.

Consistent with resistin mRNA, intracellular resistin protein appeared to be enhanced by tunicamycin in THP-1 cells at 24 h. Paradoxically, resistin protein secretion was decreased by tunicamycin in these cells. Most recently, Suragani *et al.*⁴¹ reported a similar discrepancy regarding tunicamycin treatment in U937 human monocytes. They also showed that resistin was retained in the ER as a possible chaperone of misfolded proteins on tunicamycin treatment, which could account for the decreased secretion of resistin. In addition, the secretion of secretory proteins, such as resistin, could be decreased by tunicamycin as a result of its blocking effect on N-linked glycosylation. Under ER stress conditions, such as diabetes and obesity *in vivo*, circulating resistin could be increased in the absence of pharmacological effects of tunicamycin.

Several limitations should be noted. First, the effects of pharmacological agents might not be limited to ER stress. Although two known ER stress inducers, tunicamycin and thapsigargin, similarly increased resistin mRNA in THP-1 human monocytes, DTT, another ER stress inducer, paradoxically decreased resistin mRNA, possibly through a different mechanism (Figure S3a,b). Second, the effect of tunicamycin on resistin mRNA was assessed only in the THP-1 human monocytes cell line. It appeared to be difficult to assess the effect of tunicamycin on

resistin mRNA in isolated human monocytes, as resistin mRNA was decreased to approximately one-fifth of its initial level in 24 h after the isolation of monocytes (Figure S5). Nagaev *et al.*⁴² also reported the decrease in resistin mRNA after isolation of human monocytes.

In summary, we have shown that ER stress induced by tunicamycin increased resistin mRNA through the PERK–ATF4–CHOP pathway in THP-1 human monocytes. How ER stress induces the expression of *RETN*, especially at the transcriptional level, and the pathophysiological roles of resistin under conditions of ER stress in humans remain unclear. Further studies will be required to clarify these points.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 | It was estimated that at least approximately 17% of the THP-1 cells overexpressed introduced genes by transfection.

Figure S2 | Calibration curves for resistin by immune complex transfer enzyme immunoassay.

Figure S3 | (a) Thapsigargin, the endoplasmic reticulum stress inducer, increased resistin messenger ribonucleic acid (mRNA) in THP-1 human monocytes. (b) DTT, another ER stress inducer, paradoxically decreased resistin mRNA in THP-1 human monocytes. ATF4, activating transcription factor 4; CHOP, CAAT/enhancer binding protein- α homologous protein; PERK, pancreatic ER eukaryotic initiation factor 2 α kinase; *RETN*, human resistin gene.

Figure S4 | Resistin messenger ribonucleic acid (mRNA) was not affected by the small interfering RNA of activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE1) or X-box binding protein 1 (XBP1), whereas their own mRNA was reduced. *RETN*, human resistin gene.

Figure S5 | Resistin messenger ribonucleic acid (mRNA) was decreased to approximately one-fifth of its initial level in 24 h after the isolation of monocytes. *RETN*, human resistin gene.