Lipopolysaccharide inhibits the expression of resistin in adipocytes

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Abstract

Resistin is an adipocytokine leading to insulin resistance. Endotoxin/lipopolysaccharide (LPS) has been reported to decrease the expression of resistin mRNA and protein in both lean and db/db obese mice, although the underlying mechanism remains unclear. Several models such as ex vivo culture of adipose tissues, primary rat adipocytes and 3T3-L1 adipocytes were used to further characterize the effect of LPS on the expression of resistin. LPS attenuated both the resistin mRNA and protein in a time- and dose-dependent manner. In the presence of actinomycin D, LPS failed to reduce the half-life of resistin mRNA, suggesting a transcriptional mechanism. The lipid A fraction is crucial for the inhibition of resistin expression induced by LPS. Pharmacological intervention of c-Jun N-terminal kinase (JNK) reversed the inhibitory effect of LPS. LPS down-regulated CCAAT/enhancer-binding protein α (C/EBP-α; CEBPA) and peroxisome proliferator-activated receptor γ (PPAR-γ; PPARG), while activation of C/EBP-α or PPAR-γ by either over-expressing these transcriptional factors or by rosiglitazone, an agonist of PPAR-γ, blocked the inhibitory effect of LPS on resistin. C/EBP homologous protein (CHOP-10; DDIT3) was up-regulated by LPS, while a CHOP-10 antisense oligonucleotide reversed the decrement of resistin protein induced by LPS. Taken together, these results suggest that LPS inhibits resistin expression through a unique signaling pathway involving toll-like receptor 4, JNK, CHOP-10 and C/EBP-α/PPAR-γ.

Key Words
- resistin
- LPS
- JNK
- C/EBP-α
- PPAR-γ
- CHOP-10

Introduction

Inflammation in adipose tissue is intimately linked with the onset and progression of insulin resistance. The link between insulin resistance and obesity is not completely understood. Recent studies suggest that insulin sensitivity is regulated by various fat-derived factors such as free fatty acids (FFA; Bluher et al. 2001), tumor necrosis factor α (TNFα; Hotamisligil 1999), angiotensin 2 (AT2; Folli et al. 1997) and resistin (Steppan et al. 2001). Furthermore, replenishment of adiponectin increases insulin sensitivity in different murine models of insulin resistance in vivo (Berg et al. 2001, Yamauchi et al. 2001).

Resistin, an adipocyte-secreted inflammatory molecule, has been implicated as the link between obesity, inflammation and insulin resistance. Resistin is exclusively secreted by adipocytes in mice (Banerjee et al. 2004), its serum levels increase as obesity develops (Steppan et al. 2001, Steppan &
Lazar 2002). Although resistin is produced by macrophages in humans rather than adipocytes (Savage et al. 2001), its role in human obesity is still controversial (Kusminski et al. 2005). A number of studies have linked elevated serum resistin levels with cardiovascular disease, implicating resistin in metabolic disease in humans as well as in mice (Burnett et al. 2006, Norata et al. 2007). A curious aspect of resistin biology is that, despite its rising serum levels, resistin mRNA levels are significantly decreased in adipose tissue in obese mice (Way et al. 2001, Rajala et al. 2004, Boucher et al. 2005).

Endotoxin/lipopolysaccharide (LPS) is a membrane component of Gram-negative bacteria that consists of three parts: a core polysaccharide, the repeating O-antigen structures and lipid A (Ulevitch & Tobias 1995, Miller et al. 2005). LPS binds and activates toll-like receptor 4 (TLR4) on mammalian cells, which induces transmembrane signal transduction. During bacterial infection, LPS elicits immune and inflammatory responses that can result in a fatal shock syndrome (Miller et al. 2005) and a series of metabolic alterations (Khovidhunkit et al. 2004) such as accelerated energy expenditure, elevated serum levels of FFAs, hypertriglyceridaemia and impaired insulin action in humans (Fong et al. 1990, Virkamaki & Yki-Jarvinen 1994) and rodents (Feingold et al. 1992, Nonogaki et al. 1994).

Although LPS has been reported to decrease the expression of resistin mRNA and protein in both lean and db/db obese mice, its underlying mechanism remains unclear. Here we reported that LPS inhibits resistin transcription by the mediation of signaling pathways involving TLR4, c-Jun N-terminal kinase (JNK), C/EBP-α enhancer-binding protein α (C/EBP-α; CEBPA)/peroxisome proliferator-activated receptor γ (PPAR-γ; PPARγ).

Materials and methods

Animals and chemicals

Sprague–Dawley rats (8 weeks old), 129 mice and db/db mice were housed in standard rodent cages and maintained in a regulated environment (24 °C, 12 h light:12 h darkness cycle with lights on at 0700 h). Regular chow and water were available ad libitum. These investigations conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Animal Care and Use Committee of Peking University.

LPS from Escherichia coli serotype O127:B8, detoxified LPS from E. coli O127:B8, diphosphoryl lipid A derived from E. coli FS83 (Rd mutant), SP600125, rosiglitazone, phenol red-free DMEM, insulin, dexamethasone, 3-isobutyl-1-methylxanthine, polymyxin B (PMB) and actinomycin D (Act D) were purchased from Sigma. Trizol reagent and the RT system were from Promega. Collagenase I was purchased from Invitrogen. Mouse anti-β-actin antibody antibodies against rat and mouse resistin were from Santa Cruz Biotechnology. Phospho-SAPK/JNK (Thr183/Tyr185) (G9), SAPK/JNK, C/EBP-α, C/EBP-β, PPAR-γ and CHOP-10 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). IRDye-conjugated affinity purified anti-rabbit, anti-mouse and anti-goat IgGs were purchased from Rockland (Gilbertsville, PA, USA).

Glucose tolerance test

For the oral glucose tolerance tests (OGTTs), mice were fasted for 16-18 h before the gastric administration of glucose (3 g/kg body weight) by gavage. Blood from the tail tip was collected at 0, 15, 30, 60, 90 and 120 min, and glucose concentrations were detected immediately with Glucotrend from Roche Diagnostics according to the manufacturer’s instructions.

Isolation and culture of primary rat adipocytes

Mature adipocytes were isolated from epididymal fat pads of Sprague–Dawley rats aged 6–8 weeks (160–200 g) as previously described (Jiang et al. 2004). Packed adipocytes were diluted in serum-free DMEM to generate a 10% (vol/vol) cell suspension. After being incubated at 37 °C for 1 h, adipocytes were treated with LPS (300–1000 ng/ml) for 4–24 h. For the inhibition experiments, primary rat adipocytes were pretreated with the indicated inhibitors for 1 h before treatment with LPS 300 ng/ml for 24 h.

Ex vivo culture of adipose tissues

The minced fat pads were diluted in serum-free DMEM. After being incubated at 37 °C for 1 h, adipose tissue fragments were treated with LPS (300–1000 ng/ml) for 4–24 h. For the inhibition experiments, rat adipose tissue fragments were pretreated with the indicated inhibitors for 1 h before stimulation with 300 ng/ml LPS for 24 h.

Cell culture and differentiation

3T3-L1 cells (American Type Culture Collection) were cultured in growth medium, high-glucose DMEM, supplemented with 10% FBS, 100 units/ml penicillin and
100 units/ml streptomycin. Two days post-confluence, cells were induced to differentiate with standard cocktail consisting of growth medium with 1 μmol/l dexamethasone, 10 μg/ml bovine insulin and 0.25 mmol/l 3-isobutyl-1-methylxanthine. After 3 days in differentiation medium, cells were treated with growth medium with 10 μg/ml bovine insulin for 3 days and then maintained in growth medium alone. Cells were considered mature adipocytes 8 days post-induction of differentiation, when treatment with LPS, SP600125, PMB or Act D were performed. All studies were performed in 3T3-L1 cells, passages 10–14.

**Plasmids and adenovirus transfection, and reporter assay**

The resistin promoter containing a 4.1 kb upstream region of rat resistin gene was generated by PCR using rat liver DNA as the template using the following primer sequences: sense 5'-TCTTACGCGTTGTGACCCCACTGAATAAGT-3', antisense 5'-AGATCTCGAGTGAGGGGCACATGGATAG-3'. The PCR product was cloned into pGL3 to create the resistin-luc promoter reporter plasmid. The cloned promoter was confirmed as position −3986 to +152 by DNA sequencing. Plasmids were transfected into 3T3-L1 cells using TurboTect Transfection Reagent (Fermentas, Lithuania, EU, USA) according to the manufacturer’s protocol. Cell lysates were harvested at the indicated time to examine the luciferase activity. The β-galactosidase activity was measured to normalize the transfection efficiency.

The adenoviruses expressing VP-PPAR-γ (Ad-PPAR-γ) and tTA (Ad-tTA) were expanded, titrated in 293 cells and purified by cesium chloride methods as described previously (Wang et al. 2002). For adenovirus-mediated gene transfer, confluent 3T3-L1 cells were exposed to adenoviral vectors at a multiplicity of infection of 100 for 48 h. Ad-tTA was co-infected to induce the tetracycline controllable expression or infected as a virus control. Infected 3T3-L1 cells were then lysed for subsequent analysis.

**Antisense treatment**

An antisense CHOP-10 oligonucleotide (CTGAGCCATA-GAAGTCTG) directed against the 50 coding region of the rat CHOP gene was synthesized by Invitrogen. Missense oligonucleotide (ACTGGCAATAAGGTCTC) served as control. It was of identical length, containing the same nucleotide composition in a scrambled form. Oligonucleotides had phosphorothioate linkage on the three terminal bases of the 5' and 3' ends. Transfection was performed using 1 μM oligonucleotides and TurboTect in vitro Transfection Reagent (Fermentas) as recommended by the manufacturer. Expression of fluorescein-labeled oligonucleotide was detected in 3T3-L1 at 24–96 h after transfection. The efficiency of CHOP-10 antisense to prevent CHOP-10 mRNA transcription was confirmed by quantitative real-time PCR (data not shown).

**RNA extraction and quantitative real-time PCR analysis**

Total RNA was isolated using the Trizol reagent. RT was performed using the RT system according to the manufacturer’s instructions. PCR was conducted in 25 μl volume LPS: 129 male mice received a daily i.p. injection of LPS (80 μg/kg body weight per day) for 7 days, and OGTT was examined. Control animals were treated with PBS injection in the same volume amount (C). n = 6, data were expressed as means ± S.E.M. and analyzed by two-way ANOVA or unpaired Student’s t-test, *P < 0.05 vs PBS. Total AUCs were calculated with y values set at zero. Chronic effect of

**Figure 1**

Improvement of OGTT by LPS. Acute effect of LPS: 129 male mice were injected (i.p.) with a single high dose (1 mg/kg body weight) (A) or low dose (80 μg/kg body weight) (B) of LPS and OGTT was analyzed 24 h later. Control animals received PBS. n = 6, data were expressed as means ± S.E.M., and analyzed by two-way ANOVA or unpaired Student’s t-test, *P < 0.05 vs PBS. Total AUCs were calculated with y values set at zero. Chronic effect of
Down-regulation of resistin by LPS in adipose tissues. (1) In vivo effects. Expression of resistin mRNA (A) and protein (B) in lean mice treated with LPS (80 μg/kg body weight per day) for 7 days. Resistin mRNA (C) and protein (D) in db/db mice treated with LPS (100 μg/kg body weight per day) for 7 days. Resistin mRNA levels are normalized to β-actin mRNA levels, and the results are mean ± S.E.M. of three independent experiments and analyzed by one-way ANOVA. *P<0.05, **P<0.01 compared with PBS group, n=6. (2) Effects in cultured adipocytes and ex vivo adipose tissues. Time-dependent response to 1000 ng/ml LPS in primary adipocytes (E), 3T3-L1 adipocytes (I) and adipose tissue fragments (L). Dose-dependent response to LPS treatment for 24 h in primary adipocytes (F), 3T3-L1 adipocytes (J) and adipose tissue fragments (M). Reversible effect of LPS on resistin expression. Adipocytes (G) and adipose tissues (N) were treated with LPS (300 ng/ml) for 8, 24, or 8 h then withdrawal of LPS for 16 h. Effect on resistin protein levels in primary rat adipocytes (H) and 3T3-L1 cells (K) treated with 1000 ng/ml LPS for 24 h. Resistin mRNA levels are normalized to β-actin mRNA levels, and the results are mean ± S.E.M. of three independent experiments and analyzed by one-way ANOVA. *P<0.05, **P<0.01 compared with PBS group; *P<0.05, **P<0.01 compared with LPS (300 ng/ml) treatment for 24 h, 8 h groups; n=6.
containing 2.5 μl of cDNA, 5 mM MgCl$_2$, 0.2 mM dNTPs, 0.2 μM each primer, 1.25 U Ampli Taq Polymerase and 1 μl of 800× diluted SYBR Green 1 stock using the Mx3000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA). The quality of RNA was validated with gel electrophoresis, while negative control used samples without RT.

Primers used in this study were: rat resistin; sense 5′-CTACATTGTGTTAGTCCTCC-3′, antisense 5′-GCTG TCAGTTATAGCCCTCC-3′; mouse resistin: sense 5′-TCCCTGCTGAACTGC-3′, antisense 5′-ACGAATGACCACGCAGTGC-3′; rat C/EBP-α: sense 5′-ACGCAGTGGCAGATGATTC-3′, antisense 5′-CTGGGGCATCGGAA-3′; mouse C/EBP-α: sense 5′-TGGACAAGAACAGCAACGAG-3′, antisense 5′-TCACTGCTGCAATCTGCAGCA-3′; rat PPAR-γ: sense 5′-GCCCTTTACCACAGTTGATTTCT-3′, antisense 5′-AGCAATGTCC-3′; mouse PPAR-γ: sense 5′-GCTG AACCGAGCTGGTCGATATCACTGGAG-3′, antisense 5′-GCTG AACCGAGCTGGTCGATATCACTGGAG-3′; mouse CHOP-10: sense 5′-GTGA TGTCGTTGCTTTTCT-3′, antisense 5′-AGCTTGCTGATATCACTGGAG-3′; mouse C/EBP-α: sense 5′-TGGACAAGAACAGCAACGAG-3′, antisense 5′-TCACTGCTGCAATCTGCAGCA-3′; rat PPAR-γ: sense 5′-GCCCTTTACCACAGTTGATTTCT-3′, antisense 5′-AGCAATGTCC-3′; mouse PPAR-γ: sense 5′-GCTG AACCGAGCTGGTCGATATCACTGGAG-3′, antisense 5′-GCTG AACCGAGCTGGTCGATATCACTGGAG-3′.

mRNA expression was quantified using the comparative cross threshold (CT) method. The CT value of the housekeeping gene (β-actin) was subtracted from the CT value of the target gene to obtain ΔCT. The normalized fold changes of targeted mRNA expression were expressed as 2$^{-\Delta\Delta CT}$, where ΔΔCT = ΔCT sample – ΔCT control. All real-time PCRs were performed in duplicate.

**Western blot analysis**

Cultured 3T3-L1 cells were quickly harvested, rinsed thoroughly with PBS, then homogenized on ice in lysis buffer (50 mM Tris–Cl, 15 mM EGTA, 100 mM NaCl, 0.1% Triton X-100 supplemented with protease inhibitor cocktail, pH 7.5). After centrifugation for 10 min at 4°C, the supernatant was used for western blot analysis. Protein concentration was measured by Bradford’s method. A total of 80 μg protein from each sample was loaded onto SDS–PAGE gel. Proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated for 1 h at room temperature with 5% BSA in Tris-buffered saline containing Tween-20, followed by incubation overnight at 4°C with primary antibodies. Specific reaction was detected using IRDye-conjugated secondary antibody for 1 h incubation and visualized using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). Quantification of image density in pixel was performed by using the NIH Image J software (NIH, Bethesda, MD, USA).

**Statistical analysis**

Data were expressed as means ± S.E.M. and analyzed by repeated-measures ANOVA, one-way ANOVA, two-way ANOVA, Student–Newman–Keuls test (comparisons between multiple groups), or unpaired Student’s t-test (between two groups) as appropriate, using GraphPad

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**Figure 3**

Lipid A moiety-dependent effect. Effects of LPS (300 ng/ml), detoxified LPS (300 ng/ml) or lipid A (300 ng/ml) on resistin levels in adipose tissue fragments (A), primary adipocytes (B) and 3T3-L1 cells (C). Effect of PMB (500 U/ml) in ex vivo culture of adipose tissues (D), primary adipocytes (E) and 3T3-L1 adipocytes (F). Resistin mRNA levels are normalized to β-actin mRNA levels, and the results are mean ± S.E.M. of three independent experiments and analyzed by one-way ANOVA. *P < 0.01 compared with PBS group; **P < 0.005; ***P < 0.01, LPS + PMB group compared with LPS group; n = 6.
Prism software (GraphPad Software Inc., San Diego, CA, USA). \( P \) value <0.05 denotes statistical significance. Pearson's correlation analysis was performed to determine the strength of the linear relationship between the expression level of resistin and improvements of glucose metabolism or insulin resistance induced by LPS expressed as total area under the curve (AUC).

**Results**

**Improvement of glucose metabolism by LPS**

At present, the effects of LPS on glucose metabolism are still controversial. To further explore the relationship between LPS and the glucose tolerance and insulin sensitivity, we evaluated the acute and chronic effect of LPS on the OGTT. As shown in Fig. 1A and B, acute administration at the doses of 1 mg/kg body weight or 80 \( \mu \)g/kg body weight significantly improved the glucose tolerance in mice. Similar results were also observed in mice received a daily i.p. injection of LPS at a dose of 80 \( \mu \)g/kg body weight for 7 days (Fig. 1C).

**Down-regulation of resistin by LPS in adipose tissues**

**in vivo, in vitro and ex vivo**

Since resistin is critically involved in the glucose tolerance induced by chronic inflammation, we next examined the effects of LPS on resistin. Hundred and twenty-nine mouse were injected intraperitoneally with LPS (80 \( \mu \)g/kg per day) or PBS for 7 days. The body weight of the LPS-treated mice had no difference with the PBS-treated mice (data not shown). Significant reduction in the mRNA and protein levels of resistin was detected after the LPS treatment (Fig. 2A and B). Similar result was also observed in the \( db/db \) obese mice treated with 100 \( \mu \)g/kg per day LPS for 7 days (Fig. 2C and D).

To investigate the direct effect of LPS on resistin expression, both primary rat adipocytes, differentiated 3T3-L1 adipocytes and rat \( ex \) vivo adipose tissues were treated with LPS. As shown in Fig. 2E, I and L, LPS treatment markedly decreased resistin mRNA levels in a time-dependent manner. Significant effect was observed as early as 4 h after LPS treatment in primary adipocytes and 3T3-L1 adipocytes and 8 h in rat \( ex \) vivo adipose tissues. The decrease of resistin mRNA evoked by LPS was dose dependent from 30 to 1000 ng/ml in primary adipocytes and 3T3-L1 adipocytes (Fig. 2F and J). Only 300 and 1000 ng/ml of LPS significantly inhibited the expression of resistin in \( ex \) vivo adipose tissues, while lower dose at 100 ng/ml demonstrated no effect (Fig. 2M). The protein levels of resistin in primary adipocytes and 3T3-L1 adipocytes were also decreased by LPS (Fig. 2H and K). To determine if the effect of LPS on resistin expression is reversible, adipocytes and \( ex \) vivo adipose tissues were treated with LPS (300 ng/ml) for 8 h, then incubated in the LPS-free culture media for another 16 h. As shown in Fig. 2G and N, LPS-induced down-regulation of resistin was reversible.
Inhibition of resistin through activation of TLR4 by the lipid A moiety of LPS

As lipid A is the active moiety ensuring the biological effects of LPS, we examined whether the lipid A fraction is crucial for reduction of resistin expression induced by LPS. Primary adipocytes, adipose tissue fragments and 3T3-L1 adipocytes were treated with LPS (300 ng/ml), detoxified LPS (E. coli O127:B8, 300 ng/ml) and diphosphoryl lipid A (E. coli F583, 300 ng/ml) for 24 h. Detoxified LPS devoid of the active lipid A part failed to inhibit the mRNA level of resistin, whereas diphosphoryl lipid A alone was sufficient to inhibit the mRNA level of resistin with the potency similar to that of LPS (Fig. 3A, B and C).

Since TLR4 is the endotoxin receptor responsible for transmembrane signaling transduction induced by LPS, we next investigated whether TLR4 mediates the effect of LPS. PMB is a peptide antibiotic that can bind and neutralize LPS, and therefore prevents the activation of TLR4 by LPS. We therefore used PMB to investigate whether TLR4 mediates the effect of LPS on resistin expression. Adipocytes or adipose tissues were pre-incubated with PMB at a dose of 500 units/ml for 1 h, then treated with LPS (300 ng/ml) for 24 h. PMB significantly attenuated the inhibitory effect of LPS on resistin expression in primary adipocytes, 3T3-L1 adipocytes and ex vivo adipose tissues (Fig. 3D, E and F).

Down-regulation of resistin by LPS through a transcriptional mechanism

An initial step in dissecting the mechanism by which LPS regulates resistin expression was to determine whether the down-regulation of resistin by LPS is transcriptional. For this purpose, primary and 3T3-L1 adipocytes were treated with 300 ng/ml LPS in the presence of 5 μg/ml of SP600125 (20 μM) significantly blocked the effect of LPS (1000 ng/ml) on resistin expression in primary rat adipocytes (C) and 3T3-L1 adipocytes (D). Resistin mRNA levels are normalized to β-actin mRNA levels, and the results are mean ± S.E.M. of three independent experiments and analyzed by one-way ANOVA. *P<0.01 compared with PBS group; #P<0.01, LPS + PMB group compared with LPS group or LPS + SP600125 group compared with LPS group; n=6.

Figure 5

JNK-dependent effect. Effects of LPS (1000 ng/ml) or lipid A (1000 ng/ml) on JNK phosphorylation in 3T3-L1 adipocytes (A). Blockade of TLR4 by PMB (500 U/ml) completely attenuated the phosphorylation of JNK induced by LPS (1000 ng/ml) in 3T3-L1 adipocytes (B). Inhibition of JNK phosphorylation by SP600125 (20 μM) significantly blocked the effect of LPS (1000 ng/ml) on resistin expression in primary rat adipocytes (C) and 3T3-L1 adipocytes. Resistin mRNA levels are normalized to β-actin mRNA levels, and the results are mean ± S.E.M. of three independent experiments and analyzed by one-way ANOVA. *P<0.01 compared with PBS group; #P<0.01, LPS + PMB group compared with LPS group or LPS + SP600125 group compared with LPS group; n=6.
the transcriptional inhibitor Act D. LPS treatment did not reduce the half-life of resistin mRNA as would have been expected if LPS reduced resistin mRNA by a post-transcriptional mechanism (Fig. 4A and B). The effect of LPS was further explored using a luciferase reporter vector (resistin-luc) driven by a large 4137-bp fragment of the resistin gene including the promoter and transcriptional start site. The resistin–luc reporter was active in mature adipocytes, but most of this activity was lost when cells were treated with 300 ng/ml LPS for 24 h (Fig. 4C). This observation further demonstrates that LPS reduces the transcription of resistin gene.

**JNK-dependent modulation of resistin by LPS and lipid A**

Since the stress-responsive JNK pathway may account for resistin synthesis and secretion, we next examined the effect of LPS on JNK phosphorylation in 3T3-L1 adipocytes. As shown in Fig. 5A, both LPS (1000 ng/ml) and lipid A (1000 ng/ml) significantly increased the phosphorylation of JNK. Pre-incubation of cells with PMB (500 U/ml) for 1 h completely blocked the JNK activation induced by LPS (Fig. 5B). SP600125 (20 μM), an inhibitor of JNK, significantly blocked the inhibition of resistin expression evoked by LPS in primary adipocytes and 3T3-L1 adipocytes (Fig. 5C and D).

**Role of CHOP-10**

CHOP-10 is actively involved in the regulation of the expression of adipokines including resistin. This protein bears significant homology to other C/EBPs and is induced by various stressors, including hypoxia and the unfolded protein response. CHOP-10 can have dominant negative levels of CHOP-10 and resistin are normalized to β-actin mRNA levels, and the results are mean ± S.E.M. of three independent experiments and analyzed by one-way ANOVA. *P < 0.05, **P < 0.01 compared with PBS group; *P < 0.05, **P < 0.01 LPS + SP600125 group compared with LPS group or LPS + antisense CHOP-10 group compared with LPS + misense CHOP-10 group; n = 6.
expression was elevated by LPS in ex vivo interactions with other C/EBPs leading to decreased C/EBP-α expression in 3T3-L1 adipocytes (Fig. 6D and E). A CHOP-10 antisense oligonucleotide blocked the decrement of C/EBP-α mRNA expression in adipose tissues (A) and in cultured 3T3-L1 adipocytes (C). Blockade of JNK phosphorylation by SP600125 (20 μM) markedly attenuated the decrement of C/EBP-α mRNA (D) and protein (E) in cultured 3T3-L1 adipocytes. Over-expression of C/EBP-α blocks the inhibitory effect of LPS on the resistin mRNA (f). mRNA levels of C/EBP-α and resistin are normalized to β-actin mRNA levels, and the results are mean ± S.E.M. of three independent experiments and analyzed by one-way ANOVA. *P<0.01 compared with PBS group; †P<0.01 LPS + SP600125 group compared with LPS group or LPS + C/EBP-α plasmid group compared with LPS + GFP plasmid group; n=6.

Role of C/EBP-α and PPAR-γ

C/EBP-α and PPAR-γ are critical for adipocyte differentiation and contribute to activation of adipocyte-specific genes such as adiponectin and resistin. Therefore, we next examined whether changes in C/EBP-α levels may also mediate the effects of LPS on resistin expression. Treatment with LPS substantially reduced C/EBP-α gene expression in ex vivo adipose tissues (Fig. 7A) and 3T3-L1 adipocytes (Fig. 7B and C). SP600125 (20 μM), a JNK inhibitor, blocked the inhibitory effect of LPS on the C/EBP-α expression in adipocytes (Fig. 7D and E). Activation of C/EBP-α by over-expression of C/EBP-α plasmid reversed the inhibitory effect of LPS on resistin (Fig. 7f).

Similarly, LPS significantly reduced PPAR-γ gene expression in ex vivo adipose tissues (Fig. 8A) and 3T3-L1 adipocytes (Fig. 8B and C). Inhibition of JNK phosphorylation by SP600125 (20 μM) significantly attenuated the LPS-induced inhibition of PPAR-γ expression in adipocytes (Fig. 8D and E). Activation of PPAR-γ by rosiglitazone (1 μM), an agonist of PPAR-γ, or by PPAR-γ adenoviral vectors reversed the decrement of resistin induced by LPS (Fig. 8F and G).

Discussion

The present study demonstrates that inflammation induced by LPS can decrease the resistin expression in adipose tissues. This conclusion is supported by the following observations: i) both acute and chronic administration of LPS improves glucose tolerance in mice; ii) LPS down-regulates resistin expression in mice, and in cultured adipose tissues and adipocytes; iii) the lipid A fraction is critical for the inhibitory effect of LPS on resistin; iv) the effect of LPS on resistin expression appears to be primarily transcriptional; v) three transcription factors including C/EBP-α, PPAR-γ and CHOP-10 mediate the effects of LPS; vi) inhibition of JNK blocks the effects of LPS on C/EBP-α, PPAR-γ and CHOP-10, therefore reverses the decrement of resistin expression. To the best of our knowledge, this is the first report demonstrating the involvement of multiple signaling pathways in the LPS-mediated modulation of resistin expression.
At present, the effect of LPS on insulin sensitivity is controversial. Chronic LPS injection has been demonstrated to decrease the insulin sensitivity (Agwunobi et al. 2000, Cani et al. 2007). However, studies by Tian et al. (2012) showed that low dose of LPS had no effect on insulin sensitivity. Our data suggest that low dose of LPS may improve the glucose tolerance. Consistent with our study, van der Crabben et al. (2009) showed that early endotoxemia increases peripheral and hepatic insulin sensitivity in healthy humans. Furthermore, studies by Nilsson et al. (2002) also indicate that postnatal endotoxin exposure results in increased insulin sensitivity and altered activity of neuroendocrine axes in adult female rats. Since significant reduction in resistin expression was observed after LPS treatment, we proposed that low dose of LPS may increase the insulin sensitivity by decreasing the expression of resistin.

Consistent with previous reports, our study demonstrates that LPS inhibits resistin protein and mRNA expression in both lean and obese mice. This observation is further confirmed by our studies using multiple in vitro models including cultured adipose tissues, primary adipocytes and 3T3-L1 cells, suggesting that LPS may directly act on the adipocytes to modulate the expression of resistin. LPS consists of a polysaccharide core, the repeating O-antigen structures, and lipid A. The lipid A domain is responsible for much of the toxicity of Gram-negative bacteria, causing fever, diarrhea and possible fatal endotoxic shock. Our study suggests that the lipid A moiety is sufficient for the inhibition of resistin expression. It is the lipid A domain that can bind and activate the TLR4, a functional receptor for LPS. In the present study, we demonstrate that neutralization of lipid A by PMB blocks the inhibitory effect of LPS on resistin expression in adipose tissues (A) and in cultured 3T3-L1 adipocytes (B). The protein level of PPAR-γ in 3T3-L1 adipocytes treated with 1000 ng/ml LPS for 24 h (C). Blockade of JNK phosphorylation by SP600125 (20 μM) markedly attenuated the decrement of PPAR-γ mRNA (D) and protein (E) in cultured 3T3-L1 adipocytes. Activation of PPAR-γ by rosiglitazone (1 μM) (F), or by over-expression of PPAR-γ (G) blocked the inhibitory effect of LPS on the resistin mRNA. mRNA levels of PPAR-γ and resistin are normalized to β-actin mRNA levels, and the results are mean ± S.E.M. of three independent experiments and analyzed by one-way ANOVA. **P<0.01 compared with PBS group; *P<0.05, LPS + rosiglitazone group compared with LPS group, ***P<0.01 LPS + SP600125 group compared with LPS group or LPS + PPAR-γ adenoviral vectors group compared with LPS + adenoviral vectors group; n=6.
adipocytes. Taking into account previous studies by Lin et al. (2000) revealing the expression of TLR4 on the adipocytes, it becomes clear that LPS may activate TLR4 to inhibit the expression of resistin in adipocytes.

TLRs are a recently identified family of membrane proteins, which recognize molecular patterns of invading pathogens. TLR4 is essential for LPS-mediated JNK activation, as ectopic expression of TLR4 causes activation of JNK (Muzio et al. 1998), while JNK activation is abolished in mice with TLR4 null-functional mutation. The activation of JNK by LPS requires MyD88, which directly associates with the cytoplasmic domain of TLRs. Our findings assure the critical role of JNK in the regulation of resistin by LPS. LPS and its functional domain lipid A moiety significantly increase the phosphorylation of JNK in adipocytes, suggesting the activation of JNK signaling. Inhibition of JNK phosphorylation blocks the effect of LPS on resistin expression.

Transcriptional factors such as C/EBP-α, PPAR-γ and CHOP-10 are well-known regulators of resistin and have been implicated in the mediation of ER stress on resistin levels (Fasshauer et al. 2001). In the present study, we demonstrate that the combination of C/EBP-α, PPAR-γ and CHOP-10 mediates the inhibitory effect of LPS on resistin expression. In response to LPS, CHOP-10 is activated in the adipocytes. Activation of CHOP-10 may therefore function through the dominant negative interactions with C/EBP-α to decrease the expression of C/EBP-α, PPAR-γ and subsequent inhibition of resistin levels. This concept is supported by evidence presented in this study. First, CHOP-10 expression is elevated by LPS in adipose tissues and adipocytes. Interference of CHOP gene with an antisense oligonucleotide blocks the effect of LPS on resistin levels. Second, levels of C/EBP-α and PPAR-γ are markedly reduced by LPS in adipose tissues and adipocytes. Activation of C/EBP-α and PPAR-γ reverses the inhibitory effect of LPS on resistin levels. Interestingly, LPS-induced alterations in C/EBP-α, PPAR-γ and CHOP-10 levels all require the phosphorylation of JNK since JNK inhibitor abolishes the changes of C/EBP-α, PPAR-γ and CHOP-10 and the subsequent decrease in resistin levels. As illustrated in Fig. 9, we therefore propose that LPS attenuates the resistin expression by the following signaling pathway: LPS-TLR4-JNK-CHOP-10-C/EBP-α/PPAR-γ.

Previous studies have reported the elevation of serum resistin levels in obese mice despite of the decrease in resistin mRNA in adipose tissue. Our study also demonstrates that LPS decreases the expression of resistin. Levels of resistin protein in primary adipocytes and differentiated 3T3-L1 adipocytes parallel the down-regulation in its mRNA. This raises the possibility that the discrepancy between adipose tissue and plasma resistin levels may not occur at the level of individual adipocytes but rather result from various global defects characteristic of obesity and insulin resistance. For example, it has been demonstrated that the development of obesity is associated with an increase in fat cell number (van Harmelen et al. 2003, Spalding et al. 2008), and therefore the net effect in obesity may be elevated resistin release into the circulation even though resistin secretion is decreased on a per-cell basis. Clearance of resistin by kidney also affects the serum concentration of resistin. A number of studies have demonstrated a negative correlation between renal function and resistin levels (Kielstein et al. 2003, Axelsson et al. 2006), suggesting that resistin may be cleared through the kidney. 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’s half-life in obesity may be increased because of oligomerization. Both mouse and human resistin can form oligomers, which can be detected in the circulation and have different biological actions and clearance rates compared with the monomer form (Patel et al. 2004, Gravelle et al. 2005). Interestingly, the tendency to oligomerization is concentration dependent (Aruna et al. 2008).

The discrepancy between resistin mRNA in adipose tissues and circulating protein levels may also result from the hyperinsulinemia associated with obesity and insulin resistance (Rajala et al. 2004). In vitro experiments have demonstrated that insulin treatment of mature adipocytes down-regulates resistin expression (Haugen et al. 2001, Shojima et al. 2002). There is evidence that insulin can potently decrease C/EBP-α expression in differentiated 3T3-L1 cells leading to decreased C/EBP-α binding target DNA sequences (MacDougald et al. 1995). This suggests that both inflammation and hyperinsulinemia may contribute to the decreased resistin mRNA levels in obesity by actions converging on C/EBP-α. Previous genetic and pharmacologic studies suggest a strong link between resistin and insulin resistance in mice (Meigs et al. 2007, Ley et al. 2009). However, the role of resistin as a modulator of metabolism in inflammatory states is not well understood. The present study therefore provides much needed insight into the pathophysiological role of resistin in various inflammatory states associated with insulin resistance, ranging from sepsis to type 2 diabetes.

In summary, we demonstrate that LPS is a potent negative regulator of resistin expression and secretion via TLR4-JNK-CHOP-10-C/EBP-α/PPAR-γ signaling pathway.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
X X and W A performed the experiments and analyzed data; all authors contributed to the design of the experiments, interpretation of data and the writing of the manuscript. X X, Y L and W Z revised the manuscript. All the authors approved the final version of the paper.

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