FEBS Letters 530 (2002) 158–162 FEBS 26631

Lipopolysaccharide increases resistin gene expression in vivo and in vitro

Shao-Chun Lu[a,b,*], Wei-Yeong Shieh[a], Chia-Ying Chen[b], Shu-Ching Hsu[a], Hui-Ling Chen[c]

[a] *Department of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, No. 1, Sec. 1, Jen-Ai Road, Taipei 100, Taiwan*
[b] *Department of Agricultural Chemistry, College of Agriculture, National Taiwan University, Taipei, Taiwan*
[c] *Hepatitis Research Center, College of Medicine, National Taiwan University, Taipei, Taiwan*

Received 8 August 2002; revised 13 September 2002; accepted 13 September 2002

First published online 26 September 2002

Edited by Jacques Hanoune

Abstract Although resistin has been thought to be an important link between obesity and diabetes, recent results do not support this hypothesis. We speculated that resistin may be involved in inflammatory processes and be induced by inflammatory stimuli. In this study, we tested whether lipopolysaccharide (LPS) induced resistin expression in rats. The results show that resistin mRNA levels in white adipose tissue and white blood cells were increased by LPS treatment. LPS also increased resistin mRNA levels in 3T3-L1 adipocytes and human peripheral blood monocytes. The results suggest that resistin is involved in insulin resistance and probably in other inflammatory responses.

Key words: Resistin; Lipopolysaccharide; Inflammation; Adipocyte; Monocyte

1. Introduction

Resistin, a 12.5 kDa cysteine-rich protein, is synthesized and secreted mainly by adipocytes. Steppan et al. [1] have shown that plasma resistin levels are significantly increased in both genetically susceptible and high fat diet-induced obese mice and that immunoneutralization of resistin improves hyperglycemia and insulin resistance in high fat diet-induced obese mice, while administration of recombinant resistin impairs glucose tolerance and insulin action in normal mice; they also showed that, in mice and 3T3-L1 adipocytes, resistin expression is down-regulated by rosiglitazone, a new anti-diabetic drug. On the basis of these results, they proposed that resistin is an important link between obesity and diabetes. However, the role of resistin in obesity and insulin resistance has not been confirmed in several recent studies. Way et al. [2] showed that resistin expression in white adipose tissues is significantly lower in several different obese mice models than in their lean counterparts, Le Lay et al. [3] demonstrated that a high fat diet reduces resistin expression in several strains of mice with different sensitivity to diet-induced obesity, and no association was found between resistin expression in adipocytes and type 2 diabetes or insulin resistance in at least three human studies [4–6].

Resistin has also been identified as a sensor of nutritional state in rats and has an inhibitory effect on adipocyte differentiation [7]. The sequence of the resistin gene is identical to that of FIZZ3, a member of an inflammatory response protein family described by Holcomb et al. [8]. FIZZ1, the first identified protein of this family, is overexpressed during allergic pulmonary inflammation [8]. While analyzing the 5′ flanking sequence of resistin, we found several potential binding sequences for nuclear factor κB (NF-κB), Ap-1, and STAT (unpublished observation). These transcription factors are responsible for the inducible expression of genes involved in immune, inflammatory, and stress responses [9,10]. In addition, resistin mRNA has been detected in mononuclear blood cells, which also take part in inflammatory reactions [4,5]. On the basis of this information, we speculated that resistin may be involved in inflammatory processes and might be induced by inflammatory stimuli. We therefore measured resistin expression in adipocytes and white blood cells during inflammation induced by lipopolysaccharide (LPS), a compound of the cell wall of Gram-negative bacteria that has been demonstrated to induce inflammatory reactions. Our results show that resistin mRNA levels were increased by both in vivo and in vitro LPS treatment.

2. Materials and methods

2.1. Animal experiments

Male Wistar rats (9 weeks old; 200–250 g), obtained from the Animal Center of the National Taiwan University (Taipei, Taiwan), were housed in groups of three in stainless cages on a 12-h light cycle (7.00–19.00 h) with free access to Purina rat chow and tap water. After removal of food for 14 h (from 19.00 to 9.00 h), the animals were randomly assigned to LPS or control groups. Rats in the LPS group were injected intraperitoneally (i.p.) with 3 mg/kg of *Escherichia coli* LPS (3 mg/ml in phosphate-buffered saline (PBS); Sigma, St. Louis, MO, USA), while control rats were injected with PBS (1 ml/kg). At the indicated time points, rats were killed by being placed in a CO_2 chamber and the epididymal white adipose tissue (WAT) was removed, immediately frozen in liquid nitrogen, and stored at −70°C.

In a subsequent study, 16 male Wistar rats (5 weeks old) were divided into two groups and assigned for 4 weeks to the control diet (Purina rat chow) or the same diet supplemented with rosiglitazone (0.012 mg/g diet) with free access to the diets and tap water. After this period, four rats in each group were injected i.p. with LPS or PBS as described above and the epididymal WAT was collected 4 h later.

All animal experimental procedures followed the 'Guide for the Care and Use of Laboratory Animals, National Science Council, Taiwan'.

2.2. 3T3-L1 adipocyte cultures

Mouse 3T3-L1 preadipocytes (ATCC, Rockville, MD, USA) were initially maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Gibco BRL, Life Tech-

*Corresponding author. Fax: (886)-2-2391 5295.
E-mail address: lsc@ccms.ntu.edu.tw (S.-C. Lu).

0014-5793/02/$22.00
PII: S0014-5793(02)03450-6

nologies, Rockville, MD, USA) under an atmosphere of 5% CO_2. One day after the cells reached confluence, differentiation was induced by replacing the normal culture medium for 2 days with DMEM supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 μM dexamethasone (Sigma), 50 μM insulin (Sigma), and 10% fetal bovine serum (FBS) (Gibco BRL), followed by the same medium but without 3-isobutyl-1-methylxanthine and dexamethasone for another 2 days. The cells were then cultured in DMEM containing 10% FBS for a further 6 days. To study the effect of LPS, the cells were changed to serum-free DMEM for 6 h, then treated with 25, 50, or75 μg/ml of LPS for 12 h.

2.3. Isolation of peripheral blood cells

Four hours after injection of the rats with LPS or PBS, blood was withdrawn from the portal vein into EDTA-containing tubes. Red blood cells (RBC) were lysed using RBC Lysis Solution (Gentra systems, Minneapolis, MN, USA) according to the manufacturer's instructions, then white blood cells (WBC) were pelleted by centrifugation, and total RNA prepared as described below.

Human peripheral blood monocytes (PBMC) were isolated from the whole blood of healthy volunteers. Briefly, heparinized blood was mixed with an equal volume of PBS, layered onto Histopaque 1077 (Sigma), and centrifuged for 30 min at $400\times g$. The interface containing mononuclear cells was collected and washed twice with RPMI 1640 medium, then the cells were resuspended at 2×10^6 cells/ml in RPMI 1640 medium and seeded into 6-well plates. After 6 h incubation, non-adherent cells were removed and the adherent cells (PBMC) used for total RNA isolation after incubation with 0–10 ng/ml of LPS for 4 h.

2.4. Analyses of resistin mRNA

Total cellular RNA in adipose tissue, 3T3-L1 adipocytes, and WBC was isolated according to the method of Chomczynski and Sacchi [11]. RNA concentrations were estimated from the absorbance at 260 nm. For Northern blot analysis, 15 μg of total RNA was electrophoresed on a 1% agarose-formaldehyde gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled cDNA probes for rat or mouse resistin in animal studies and 3T3-L1 cell line studies, respectively. The blot was then hybridized with a probe for 18S RNA to check the amount of RNA loaded on the gel.

Levels of resistin mRNA in human PBMC were determined by RT-PCR. Briefly, 4 μg of RNA was annealed with 1 μl of oligo-dT primer (10 μM) and reverse transcribed with 2 units of MMLV reverse transcriptase at 42°C for 60 min in a final volume of 20 μl. For the PCR reaction, 2 μl of the reverse transcription mixture was used for each reaction. Resistin mRNA was amplified for 43 cycles using the forward primer 5′-TCTAGCAAGACCCTGTGCTCCA-3′ and the reverse primer 5′-CTCAGGGCTGCACACGACAG-3′, designed according to Steppan et al. [1]. Amplification of GAPDH mRNA for 18 cycles using the forward primer 5′-AAAGGATCCACTGGCGTCTTCACCACC-3′ and the reverse primer 5′-GAATTCGTCATGGATGACCTTGGCCAG-3′ was used as the internal control. The PCR products were separated on a 1% agarose gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled cDNA probes for human resistin or GAPDH.

A 509-bp rat resistin cDNA fragment, a 517-bp mouse resistin cDNA fragment, and a 1297-bp rat 18S rRNA cDNA fragment were amplified by RT-PCR using the following primers: rat resistin (forward 5′-TGTGCCCTGCTGAGCTCTCT-3′; reverse 5′-CAGACCCTCATCTCGTTTCCT-3′) [7], mouse resistin (forward 5′-GGAGCTGTGGGACAGGAGCTA-3′; reverse 5′-ACGTGAGTGCAGGTGCCTGTA-3′) [1], and rat 18S rRNA (forward 5′-TACCTGGTTGATCCTGCCAG-3′; reverse 5′-TGTCAATCCTGTCCGTGTCC-3′) [12]. The amplified DNA fragments were cloned into a pGEM-Teasy vector (Promega, Madison, WI, USA), and sequenced to confirm their identity. The cDNA fragments were labeled with [^{32}P]dCTP (NEN Life Science Products, Boston, MA, USA) and used as probe for Northern or Southern blot analyses. The signals of resistin, GAPDH and 18S RNA were quantified by a phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA).

2.5. Statistical analysis

Results are expressed as the means ± S.D. Comparisons were made using Student's *t*-test. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. LPS increased resistin mRNA expression in epididymal adipose tissue in vivo

When rats were fasted for 14 h, then injected i.p. with LPS (3 mg/kg BW), levels of resistin mRNA in epididymal WAT increased in a time-dependent manner (Fig. 1). A significant ($P<0.05$) 37% increase in resistin mRNA levels was detected 1 h after LPS injection, while maximal levels (113% increase; $P<0.01$) were seen 8 h after injection (Fig. 1).

3.2. Rosiglitazone treatment reduces resistin mRNA levels, but does not prevent the LPS-induced increase in resistin mRNA

Since resistin can be down-regulated by rosiglitazone [1], we tested whether rosiglitazone could prevent this LPS-induced increase in resistin mRNA. Rats were fed normal chow or chow containing rosiglitazone for 4 weeks, then injected as above and killed 4 h later. As in the previous experiment, LPS treatment of control chow-fed rats resulted in a 66% increase in resistin mRNA levels in WAT (lane 2 vs. lane 1; $P<0.001$) (Fig. 2). Although resistin mRNA levels in rosiglitazone-fed rats were only 40% those in the corresponding controls (lane 3 vs. lane 1 and lane 4 vs. lane 2; $P<0.001$), LPS injection still resulted in a 66% increase in adipose resistin mRNA (lane 4 vs. lane 3; $P<0.05$) (Fig. 2). However, resistin mRNA levels in LPS-injected rosiglitazone-fed rats were only 66.5% of those seen in untreated control rats (lane 4 vs. lane 1; $P<0.005$).

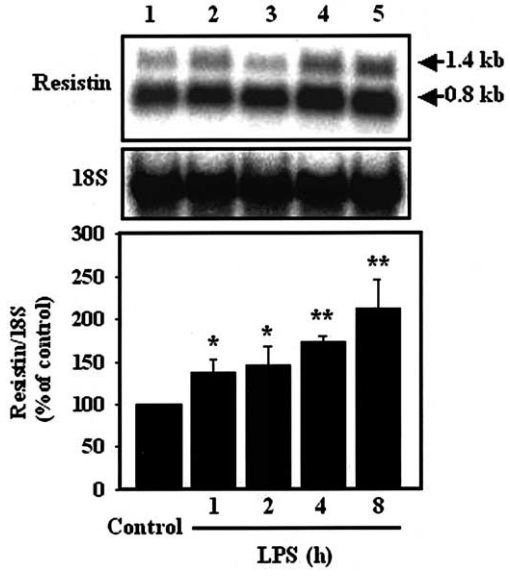

Fig. 1. Resistin mRNA levels in rat epididymal WAT are increased by in vivo LPS treatment. Fasting rats were injected i.p. with PBS (control) or LPS (3 mg/kg BW) and total RNA was isolated from epididymal WAT 1, 2, 4, or 8 h after injection. Resistin mRNA was detected by Northern blotting, as described in Section 2. Upper panels: Representative Northern blot results. Lower panels: Resistin mRNA levels normalized to those for 18S mRNA and expressed relative to those in the control (relative value = 100%); the results are the mean ± S.D. for three rats. *$P<0.05$; **$P<0.01$ compared to the control.

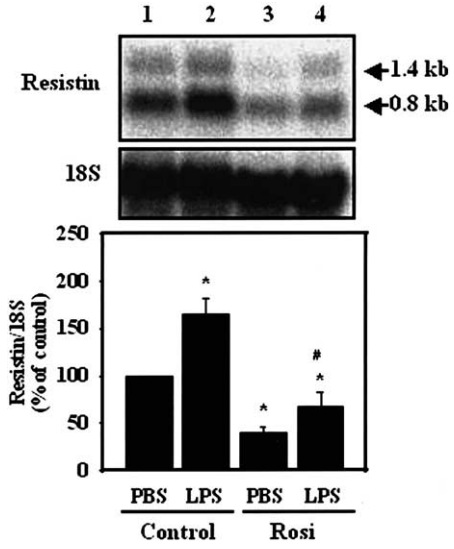

Fig. 2. Effect of rosiglitazone on WAT resistin mRNA expression in control and LPS-injected rats. Rats were fed Purina rat chow (lanes 1 and 2) or the same diet containing rosiglitazone (0.012 mg/g diet) (lanes 3 and 4) for 4 weeks. Four rats in each group were then injected with PBS (lanes 1 and 3) or LPS (3 mg/kg BW; lanes 2 and 4) and killed 4 h after injection. Levels of resistin mRNA in epididymal WAT were detected and the results expressed as described in Fig. 1; the values are the mean ± S.D. for four rats. $^{*}P < 0.005$ compared to control-fed/PBS-injected rats (lane 1); $^{\#}P < 0.05$ compared to rosiglitazone-fed/PBS-injected rats (lane 3).

3.3. *LPS increases resistin mRNA expression in 3T3-L1 adipocytes*

When 3T3-L1 adipocytes were treated for 12 h with various concentrations of LPS (0–75 μg/ml), a dose-dependent increase in resistin mRNA levels was seen (Fig. 3), with an approximately 120% increase using 75 μg/ml of LPS.

3.4. *Resistin is expressed in rat WBC and is upregulated by LPS in vivo*

To examine the expression and response of resistin mRNA to LPS stimulation in WBC, rats were injected with 3 mg/kg BW of LPS (three rats) or PBS (control, two rats), then resistin mRNA levels in WBC were measured 4 h later by Northern blot analysis. Resistin mRNA was detectable in WBC from control rats (lanes 1 and 2, Fig. 4), but the levels were significantly lower than those in adipose tissue (data not shown). However, a significant increase in resistin mRNA

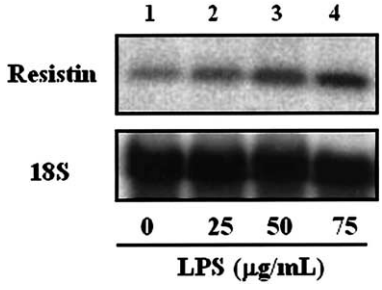

Fig. 3. LPS-induced resistin mRNA expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes in serum-free medium were incubated for 12 h with a final concentration of 0, 25, 50 or 75 μg/ml of LPS, then resistin mRNA was detected by Northern blotting as described. Similar results were obtained in two independent experiments.

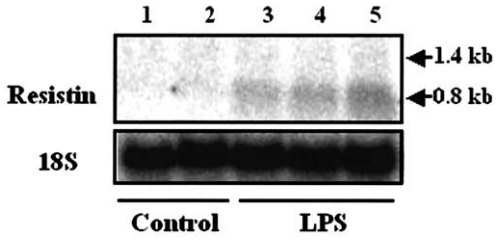

Fig. 4. Resistin mRNA levels in rat WBC are increased by in vivo LPS treatment. Fasting rats were injected with PBS (two rats, lanes 1 and 2) or LPS (three rats, lanes 3–5) as described in Section 2, then, 4 h later, WBC were isolated and resistin mRNA assayed by Northern blotting as described.

levels (175% of control levels), mainly in the 0.8-kb mRNA, was detected in LPS-treated rats (lanes 3–5, Fig. 4). This result shows that, as in adipose tissue, resistin mRNA in WBC was up-regulated by LPS.

3.5. *Resistin expression in human PBMC is upregulated by LPS in vitro*

Since resistin is also expressed in mononuclear cells, we tested the effect of LPS on resistin expression in human PBMC in vitro. When monocytes from three healthy volunteers were briefly cultured, then treated for 4 h with 0, 1, 5, or 10 ng/ml of LPS, resistin mRNA levels were increased by LPS in a dose-dependent manner (Fig. 5). A 220% increase in resistin mRNA levels was seen in cells treated with 1 ng/ml of LPS (lane 2), and maximal expression (a 460% increase) was seen at 10 ng/ml LPS (lane 4). The response to LPS induction varied slightly in monocytes from different volunteers, but all three samples showed an obvious increase after LPS treatment.

4. Discussion

The present study shows that LPS increased resistin mRNA levels in rat WAT and WBC in vivo and in mouse 3T3-L1 adipocytes and human PBMC in vitro. LPS administration, a widely used experimental model of infection and inflammation, induces hyperglycemia and insulin resistance in rats

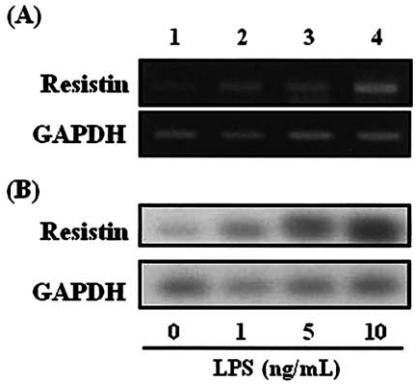

Fig. 5. Resistin mRNA levels in cultured human PBMC with or without LPS treatment. Human blood monocytes, isolated as described in Section 2, were treated for 4 h with 0, 1, 5, or 10 ng/ml of LPS. Resistin mRNA was detected by RT-PCR (A), then transferred to a nylon membrane and probed with a ^{32}P-labeled cDNA probe (B). Cells from three individuals were tested, with similar results.

[13,14] and humans [15]. However, the mechanism of LPS-induced insulin resistance is still poorly understood. Since resistin has been identified as a signaling molecule that can impair glucose tolerance and insulin action [1], our results suggest that it may be involved in insulin resistance during LPS-induced inflammation. During the submission of this manuscript, Rajala et al. [16] reported down-regulation of resistin in adipose tissue of FVB mice upon LPS treatment. The conflicting results could be due to different doses or periods of LPS treatment, or the use of a different animal model. In Rajala's study, FVB mice were injected with 100 ng/g BW LPS and resistin response was evaluated 24 h post injection, while in our study, Wistar rats were injected with 3 mg/kg BW LPS for 1, 2, 4 or 8 h. As shown in Fig. 1, resistin mRNA increased as early as 1 h after LPS injection and the highest increase was seen at 8 h. We also observed an 86% increase of adipose resistin mRNA when C57BL/6J mice were injected with 3 mg/kg LPS for 4 h (unpublished data).

Systematic administration of LPS is known to increase the release of several cytokines and other cellular mediators, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6, from the peripheral immune system via activation of NF-κB, Ap-1, and/or STAT [9,10]. It is possible that LPS induces resistin expression in monocytes via one of these pathways. However, the mechanism by which LPS induces gene expression in adipocytes is less well understood. Kapur et al. [17] have shown that expression of inducible nitric oxide synthase in 3T3-L1 adipocytes is not induced by LPS alone, but is induced by the combination of LPS, TNF-α, and interferon-γ (IFN-γ). Grunfeld et al. [18] have shown that induction of leptin expression in adipose tissue by LPS can be mimicked by IL-1 and/or TNF-α. Our study showed that LPS alone, although at a relatively high concentration (75 μg/ml), was able to increase resistin mRNA levels in 3T3-L1 adipocytes. Whether IL-1, IL-6, and/or IFN-γ are also involved in resistin regulation remains unclear. Apparently LPS-induced resistin expression is independent of TNF-α, since TNF-α lowers resistin mRNA levels in 3T3-L1 adipocytes [19]. The mechanism whereby LPS stimulates resistin expression in adipocytes remains to be established. Our in vitro studies showed that 3T3-L1 adipocytes and isolated human PBMC respond to LPS with increased resistin mRNA expression (Figs. 3 and 5). These in vitro systems may provide a useful model for studying the regulation of resistin gene expression.

Regardless of the mechanism by which LPS stimulates resistin expression, increased resistin levels are likely to have important physiological consequences. Two recent human studies showed higher resistin mRNA levels in mononuclear blood cells than in isolated adipocytes [4,5]. In this study, we further demonstrated that resistin expression in human PBMC was stimulated by LPS (Fig. 5), resistin mRNA levels being increased more than two-fold by 4 h treatment with 1 ng/ml of LPS and 5.6-fold using 10 ng/ml of LPS. This effect could be of physiological importance in man. These characteristics of resistin are striking similar to those of TNF-α and IL-6, two other proinflammatory cytokines expressed in both adipocytes and monocytes [20]; furthermore, all three factors have important effects on lipid and glucose metabolism [21] and are involved in obesity-related insulin resistance [1,22]. In addition, resistin belongs to the FIZZ inflammatory protein family [8]. Taken together, these results suggest that, like TNF-α and IL-6, resistin may participate in inflammatory processes.

In agreement with the results of Steppan et al. [1], rosiglitazone decreased resistin mRNA levels by 60% in the WAT of non-obese Wistar rats (lane 3, Fig. 2). However, it markedly increased the mass of the epididymal WAT by 53% compared to control rats (data not shown), this effect being attributable to the fact that it activates the peroxisome proliferator-activated receptor (PPAR)-γ and induces adipocyte differentiation. Several recent studies suggest that PPAR agonists may modulate LPS-induced cytokine production [9,23,24]. However, rosiglitazone did not inhibit LPS-induced resistin expression in rats, since LPS caused a 65% increase in resistin mRNA levels in both control and rosiglitazone-treated rats (Fig. 2). These results suggest that LPS and rosiglitazone regulate resistin expression through different pathways.

Resistin has been reported to be expressed exclusively in adipocytes [1], but the present study showed that it was also expressed in rat WBC and human monocytes. We also demonstrated that resistin mRNA expression was up-regulated by LPS in these cells. In addition, we observed that resistin mRNA was detectable in THP-1 cells (a human monocytic leukemia cell line) and in Raw264.7 cells (a mouse macrophage cell line) and that its levels in these cells could be increased by LPS or phorbol myristate acetate (unpublished data). Our findings provide evidence that resistin may be involved in insulin resistance and probably in other inflammatory responses. The precise physiological function of resistin during inflammation remains unclear and deserves further investigation.

Acknowledgements: This work was supported by Research Grant NSC91-2320-B-002-145 from the National Science Council of Taiwan.

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