EXPERIMENTAL STUDY

Resistin promotes 3T3-L1 preadipocyte differentiationHaixia Gong^{1,2}, Yuhui Ni^{1,2}, Xirong Guo^{1,2}, Li Fei², Xiaoqin Pan², Mei Guo² and Ronghua Chen²¹Department of Pediatrics, the second affiliated hospital of Nanjing Medical University, 121 Jiangjiayuan Road, Nanjing, 210011, People's Republic of China and ²Laboratory of Pediatrics Medical, Center of Human Functional Genomics, Nanjing Medical University, 140 Hanzhong Road, Nanjing, 210029, People's Republic of China

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Abstract

Objective: To investigate the relationship between resistin (a potential link between obesity and type 2 diabetes) and preadipocyte differentiation.

Design: A rat resistin expression vector was transfected into 3T3-L1 preadipocytes and differentiation was compared between normal 3T3-L1 cells, rat resistin-transfected cells and non-transfected cells grown in conditioned medium taken from resistin-expressing cultures.

Methods: The rat resistin gene was inserted into the pDual GC and pEGFP-N2 expression vectors for examination of the effects of resistin overexpression in 3T3-L1 cells before and after differentiation was stimulated with 3-isobutyl-1-methylxanthine (MIX), insulin and dexamethasone (DEX). Smaller conserved fragments were inserted into short interference RNA (siRNA) expression vectors, for examination of the effect of targeted resistin inhibition on differentiation of resistin-overexpressing 3T3-L1 cells.

Results: Prior to stimulation, the resistin-transfected 3T3-L1 cells contained many more small lipid droplets than did non-transfected 3T3-L1 cells. Following stimulation, differentiation in the resistin-transfected 3T3-L1 cells was dramatically promoted, especially in the early stages. Stimulation of differentiation was also observed in non-transfected 3T3-L1 cells grown in resistin protein-containing conditioned medium. The expression of adipocyte differentiation-associated markers such as CCAAT enhancer binding protein (C/EBP α), retinoid X receptor (RXR α) and lipoprotein lipase (LPL) was upregulated in resistin-overexpressing cells, whereas expression of preadipocyte factor-1 (Pref-1), an inhibitor of preadipocyte differentiation, was downregulated. In addition, expression of two of the three tested siRNAs inhibited the adipogenesis process, providing further evidence that resistin promotes the differentiation of preadipocytes to adipocytes.

Conclusion: Resistin can promote preadipocyte differentiation. Based on this, we propose that resistin may be an important candidate mediator of obesity-induced insulin resistance.

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Adipocytes secrete a number of molecules that are capable of influencing cellular responsiveness to insulin (1, 2). Resistin, a novel cysteine-rich secretory signaling molecule, was recently identified as a member of the diverse family of adipocyte-derived polypeptides that may link obesity and type 2 diabetes mellitus (3). Serum resistin levels were markedly increased in both diet-induced and genetically obese mice; these levels were decreased by treatment with rosiglitazone, an anti-diabetic drug that can enhance insulin sensitivity (3). Resistin treatment of normal mice impaired glucose tolerance, whereas immunoneutralization of the resistin protein in mice increased insulin sensitivity (3). Moreover, resistin is expressed mainly in adipose tissues, and was found to be significantly upregulated

during the differentiation of preadipocytes to mature adipocytes (4). Surprisingly, both resistin and resistin-like molecules have been reported to inhibit preadipocyte differentiation (4, 5). Overall, these findings suggest that resistin plays a role in preadipocyte differentiation, and may be involved in the molecular mechanisms by which increased adiposity causes insulin resistance and type 2 diabetes.

To explore the function of resistin in the process of preadipocyte differentiation (adipogenesis), we cloned the rat resistin gene into the pDual GC vector for expression of a resistin fusion protein containing a thrombin cleavage site, three c-myc epitope tags and a 6 $\times$ His tag. This construct was stably transfected into 3T3-L1 cells, which were found to differentiate much more quickly than non-transfected 3T3-L1 cells. We hypothesized that secreted resistin inhibits

$$\begin{array}{c} \downarrow \\ 5' - \text{N}_{-2} \text{N}_{-1} \text{CTCTTCN}_x \text{N}_1 \text{N}_2 \text{N}_3 \text{N}_4 \cdots \cdots 3' \\ 3' - \text{N} \text{N} \text{GAGAAGN} \text{N} \text{N} \text{N} \text{N} \text{N} \cdots \cdots 5' \\ \uparrow \end{array}$$

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untreated or resistin-transfected 3T3-L1 cells, which were then grown in six-well culture plates with complete culture medium. Lipopolysaccharide (LPS)-free vector (2 μ g per well) was used for each transfection; the ratio of Fugene 6 to vector was 6:1. Two days (48 h) after transfection, cells and supernatants were collected for RT-PCR and Western blot detection.

RNA preparation and amplification by RT-PCR

Total RNA was isolated from 3T3-L1 cells using the TRIZOL method (Invitrogen). One microgram of total RNA was converted to cDNA using 200 U Moloney murine leukemia virus RT (Promega), and an aliquot (10%) of the resulting cDNA was amplified with the primers listed in Table 1. The number of cycles and reaction temperatures used in the semiquantitative RT-PCR assay were optimized to provide a linear relationship between the amount of input template and the

Five pSilencer 2.0-U6 vectors including a negative and a positive control were Fugene 6-transfected into

Table 1 Primers for RT-PCR amplification.

FAS, fatty acid synthetase; C/EBP α , CCAAT enhancer binding protein; Pref-1, preadipocyte factor-1; LPL, lipoprotein lipase; RXR α , retinoid X receptor; R, reverse primer; F, forward primer; T_a , annealing temperature.

amount of PCR product generated over a concentration range from 0.5 to 10 µg total RNA.

Western blot analysis

Cultured supernatants of pDual GC-resistin-transfected 3T3-L1 cells and pSilencer 2.0-U6-GAPDH-transfected 3T3-L1 cells were collected. Total proteins were isolated from these cells using the TRI_{ZOL} method (Invitrogen), and the protein concentration was determined by BCA assay (Pierce, USA). Total protein (1 µg) was subjected to 12% SDS-PAGE, electro-blotted onto PROTRAN nitrocellulose transfer membranes (Schleicher and Schuell, Germany), and immunodetected using mouse anti-His6 antibody (Clontech) and goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate (Amersham) with an ECL Western blot analysis system (Amersham).

Results

Resistin stimulates preadipocyte differentiation

Consistent with the previous report by Kim *et al.* (4), resistin mRNA was barely detected in normal,

non-differentiating 3T3-L1 cells, and was markedly increased during differentiation (Fig. 3). The expression of fatty acid synthetase (FAS) was also upregulated (Fig. 3). In cells transfected with pDual GC-resistin, rat resistin mRNA and protein were expressed at higher levels throughout differentiation (Figs 3 and 4), although mouse resistin mRNA was barely detectable (data not shown). In addition, the differentiation process of rat resistin-overexpressing 3T3-L1 preadipocytes was significantly quickened. Compared with non-transfected 3T3-L1 cells, many more lipid droplets were visible in transfected cells prior to stimulation (day 0), and the degree of cellular contraction decreased after cells were stimulated with differentiation-inducing reagents. The transfected cells underwent extensive adipocyte conversion as judged by lipid staining using Oil Red O (Fig. 5). Consistent with the observed promotion of differentiation, the expression of several adipocyte differentiation markers such as CCAAT enhancer binding protein (C/EBPα), retinoid X receptor (RXRα) and lipoprotein lipase (LPL) was also upregulated at the onset of differentiation in rat resistin-transfected 3T3-L1 cells. Similarly, expression of preadipocyte factor-1 (Pref-1), an inhibitor of adipocyte differentiation, was downregulated, and the expression of glucose transporter-4 (GLUT4) remained relatively consistent (Fig. 6).

Figure 3 Resistin and FAS mRNA expression during preadipocyte differentiation. PDual GC-resistin-transfected or non-transfected 3T3-L1 cells were induced to differentiate into adipocytes as above. Total RNA (1 µg) was reverse transcribed and an aliquot (10%) of the resulting cDNA was PCR amplified, electrophoresed in an ethidium bromide agarose gel and visualized under u.v. light. 1, day 0 of differentiation; 2, day 2 of differentiation; 3, day 4 of differentiation; 4, day 6 of differentiation; 5, day 8 of differentiation.

Figure 4 Recombinant resistin is secreted mainly as a trimer. 3T3-L1 preadipocytes were transfected with pDual GC-resistin. Stably expressing lines were selected by G418 and differentiation was induced by treatment with MIX, insulin and DEX. Medium collected from these cells was electrophoresed on 12% SDS-PAGE gels and recombinant protein expression was detected by Western blotting with anti-His antibody. M, marker; 1, day 0 of differentiation; 2, day 2 of differentiation; 3, day 4 of differentiation; 4, day 6 of differentiation; 5, day 8 of differentiation.

Figure 5 Resistin stimulates preadipocyte differentiation. 3T3-L1 preadipocytes were induced to differentiate by treatment with DMEM plus 15% FBS containing 10 μ g/ml insulin, 0.5 mM MIX and 1 μ M DEX. During the differentiation process (day 0, day 4 and day 8), the cells were fixed and stained with Oil Red O to visualize lipids. (A) Untreated 3T3-L1 cells; (B) pDual GC-resistin-transfected 3T3-L1 cells; (C) pDual GC-resistin- and pSilencer 2.0-U6-resistin₁-cotransfected 3T3-L1 cells. 1, day 0 of differentiation; 2, day 4 of differentiation; 3, day 8 of differentiation.

Subcellular localization of resistin

As the Proscan program predicted the presence of a leucine zipper motif in the rat resistin protein, we next questioned whether resistin acts as a transcription factor to stimulate preadipocyte differentiation during adipogenesis. To address this, we investigated the subcellular localization of rat resistin-enhanced GFP (EGFP) prior to and during differentiation of resistin-transfected 3T3-L1 cells. We identified faint fluorescence in the cytoplasm at all time points before and during 3T3-L1 differentiation; most of the resistin was secreted into the cell culture medium (Fig. 7).

Resistin exists mainly as trimers

Resistin was believed to exist mainly as disulfide-linked homodimers (11), or to form dimers that then associated with each other to form tetramers, hexamers, octamers and so on (12). Our results indicate that, under our experimental conditions, exogenous resistin mainly exists as trimers, as determined by comparison to a standard molecular weight marker (Fig. 4). When sample loading was increased from 1 to 15 μ g, we were able to detect monomeric, dimeric, tetrameric and pentameric resistin forms (Fig. 4).

siRNA inhibition of resistin expression inhibits preadipocyte differentiation

Contrary to the previous report by Kim *et al.* (4), we found that resistin promoted 3T3-L1 preadipocyte differentiation. To further verify our results, we inhibited resistin expression in 3T3-L1 cells via transfection of resistin-specific siRNA expression vectors. As shown in Fig. 8, the pSilencer 2.0-U6-resistin₁ and pSilencer 2.0-U6-resistin₃ vectors remarkably downregulated the expression of rat and mouse resistin at both the mRNA and protein levels, while no significant effects were detected in cells transfected with pSilencer 2.0-U6-resistin₂. These results were consistent with the expression of FAS mRNA and the differentiation process of normal or resistin-transfected 3T3-L1 cells.

Discussion

Just as transgenic and knockout models allow researchers to study gene function in vivo, overexpression and RNA interference are powerful technologies for exploring gene function in mammalian cells. Recent studies in murine models have suggested that resistin may represent the long-sought link between obesity and insulin resistance (2, 3). Resistin was originally identified as a

Figure 6 The expression of several adipocyte differentiation markers at the onset of differentiation in non-transfected or rat resistin-transfected 3T3-L1 cells. At the onset of differentiation, the expression of C/EBP α , RXR α and LPL in rat resistin-transfected 3T3-L1 cells was upregulated, whereas the expression of Pref-1 was downregulated and there was no detectable change in the level of GLUT4. 1, day 0 in rat resistin-transfected 3T3-L1 cells; 2, day 0 in non-transfected 3T3-L1 cells; 3, day 2 in rat resistin-transfected 3T3-L1 cells; 4, day 2 in non-transfected 3T3-L1 cells.

gene that is negatively regulated by several kinds of troglitazones (TZDs), insulin-sensitizing drugs, and is significantly upregulated during preadipocyte differentiation to mature adipocytes (3, 13). Resistin, which is mainly expressed in adipose tissue and is upregulated in several obese models, was thought to promote preadipocyte adipogenesis directly. However, unexpected reports indicated that both resistin and resistin-like molecule α (RELM α) inhibit adipocyte differentiation (4, 5). To address this apparent contradiction, we used *in vitro* overexpression and RNA interference experiments to explore the effect of resistin on preadipocyte differentiation.

Compared with non-transfected 3T3-L1 cells, we found that pDual GC-resistin-transfected cells exhibited much more extensive adipogenesis. This promotion of adipogenesis was evident even at the very early stages of differentiation; small lipid droplets could be

observed in transfected cells as early as day 0. This notable increase in adipogenesis was consistent throughout the whole process of differentiation.

To further confirm that resistin promotes adipogenesis, we generated short (21–23 nucleotide) synthetic double-strand siRNAs, which have been shown to induce significant, though transient, sequence-specific silencing effects (6, 7, 14). To prolong the effect, we synthesized three pairs of rat and mouse resistin mRNA-specific hairpin oligonucleotides and inserted them into the pSilencer 2.0-U6 siRNA expression vector. We found that transfection of pSilencer 2.0-U6-resistin₁ and pSilencer 2.0-U6-resistin₃ significantly downregulated resistin and FAS expression in both normal and resistin-transfected 3T3-L1 cells. These cells showed inhibition of the resistin-mediated increase in differentiation. However, adipogenesis in resistin-transfected cells remained greater than that observed in normal 3T3-L1 cells, suggesting that the silencing was incomplete. It is not clear why pSilencer 2.0-U6-resistin₂ did not show significant silencing effects. The secondary structure of the target mRNA does not appear to have strong influence on silencing (9, 15), but it is possible that nearby regulatory protein binding sites could have interfered with binding of the siRNA or the RNA-induced silencing complex (RISC).

The mechanisms by which resistin regulates adipocyte differentiation are still unclear. Resistin is a secretory protein that is probably transported in the bloodstream, where it may interact with receptors on the surface of target cells such as hepatocytes and myocytes (2). However, the *in vitro* experiments lack both bloodstream and target organs, suggesting that adipocytes may be direct targets of resistin. This could occur through one of two mechanisms: (1) the adipocyte cell membrane contains a receptor capable of binding autosecreted resistin and thereby triggering signal transduction cascades that promote preadipocyte differentiation; or (2) resistin, which contains a putative leucine zipper, acts as a transcription factor to regulate the expression of genes associated with energy metabolism or preadipocyte proliferation and differentiation. To investigate these hypotheses, we examined the subcellular localization of resistin by examining cells transfected with recombinant resistin-EGFP protein. We did not detect resistin in the nucleus of differentiating adipocytes and found that the majority of the protein was secreted. Thus, it seems more likely that the adipocytes may be able to respond to extracellular signaling from secreted, bound resistin. To provide further validation for this, normal 3T3-L1 cells were cultured in conditioned medium collected from cells stably expressing pDual GC-resistin. We observed an increase in differentiation under these conditions (data not shown), providing further evidence that secreted resistin increases adipocyte differentiation *in vitro*.

Figure 7 Subcellular location of resistin before and after induction of differentiation in 3T3-L1 cells. The full-length coding region of the rat resistin gene was inserted into the pEGFP-N2 vector, and stably transfected into 3T3-L1 cells. Faint fluorescence was observed in the cytoplasm before and during 3T3-L1 differentiation. (A) pEGFP-N2 plasmid-transfected 3T3-L1 cells; (B) pEGFP-N2-resistin-transfected 3T3-L1 cells before differentiation; (C) pEGFP-N2-resistin-transfected 3T3-L1 cells, day 0 of differentiation; (D) pEGFP-N2-resistin-transfected 3T3-L1 cells, day 2 of differentiation.

Figure 8 Silencing of the resistin gene in non-transfected or rat resistin-transfected 3T3-L1 cells. A pair of reverse complementary cDNA fragments derived from the mouse GAPDH gene served as the positive control, while insert-free pSilencer 2.0-U6 vector served as the negative control. The concentration of GAPDH protein in normal and resistin-transfected 3T3-L1 cells was significantly decreased 48 h after transfection with the pSilencer 2.0-U6-GAPDH construct (C). Compared with the negative control, transfection with the pSilencer 2.0-U6-resistin₁ and pSilencer 2.0-U6-resistin₃ vectors downregulated rat and mouse resistin expression at both the mRNA and protein levels (A and B). In contrast, no significant effects were detected in cells transfected with the pSilencer 2.0-U6-resistin₂ vector. These results were consistent with the expression of FAS mRNA. 1, day 0 of differentiation; 2, day 2 of differentiation; 3, day 4 of differentiation; 4, day 6 of differentiation; 5, day 8 of differentiation.

In summary, our data support the hypothesis that resistin stimulates preadipocyte differentiation. Thus, the protein may serve as a link between obesity and insulin resistance. Further studies will be needed to explore the regulation and function of resistin at the gene and protein levels, in the hope that a better understanding of this protein may lead to new therapies for diabetes mellitus 2.

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