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Resistin does not down-regulate the transcription of insulin receptor promoter*

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Abstract: Objective: To detect the effect of resistin on the transcription of insulin receptor promoter. Methods: Luciferase reporter gene was fused downstream of human insulin receptor promoter and the enzymatic activity of luciferase was determined in the presence or absence of resistin. The resistin expressed with plasmid was stained with antibody against Myc tag which was in frame fused with resistin coding sequence, and then imaged with confocal microscopy. Results: The treatment of pIRP-LUC transfected cells with recombinant resistin did not result in significant difference in the enzymatic activity of luciferase compared to the untreated cells. Cell staining showed that green fluorescence could be observed in the cytoplasm, but not in the nucleus. Conclusion: The results suggest that the endogenous resistin may functionally locate in the cytoplasm, but does not enter the nucleus and not down-regulate the transcription of insulin receptor promoter.

Key words: Resistin, Insulin receptor promoter, Luciferase reporter gene
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INTRODUCTION

Type 2 diabetes (T2D) is a condition that is expected to affect over 300 million people worldwide by 2025 (Adeghate *et al*., 2006). Although T2D has a multi-factorial etiology, one of the well-established risk factors for T2D is obesity (Proietto, 2005). Recently, adipose tissue was considered to not only act as an energy reservoir, but also emerge as a key secretary organ (Kershaw and Flier, 2004), releasing a number of bioactive molecules such as leptin, adiponectin, tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor type 1, and the recently described hormone resistin (Steppan *et al*., 2001a).

Resistin, a cysteine-rich hormone belonging to the newly discovered resistin-like molecule (RELM) or found in the inflammatory zone (FIZZ) family (Holcomb *et al*., 2000; Kim *et al*., 2001), has been proposed to form a biochemical link between obesity and T2D (Steppan *et al*., 2001a; 2001b). In vivo and in vitro studies have demonstrated that high concentrations of resistin impair insulin sensitivity and cause insulin resistance. In the diet-induced obese mice, immunoneutralization of resistin led to a 20% drop in blood glucose, and improved insulin sensitivity (Muse *et al*., 2004). In contrast, recombinant resistin impairs the insulin-stimulated glucose uptake (Banerjee *et al*., 2004). Moreover, overexpression of resistin led to inhibition of adipocyte differentiation (Rajala *et al*., 2003) and impaired glucose tolerance in hepatocytes (Zhou *et al*., 2006), implying that resistin induces insulin resistance. However, the mechanism of resistin in inducing insulin resistance remains poorly understood. It was reported that resistin down-regulated mRNA and protein expression of insulin receptor in rodent pancreatic beta-cells, but did not affect insulin secretion (Brown *et al*., 2007).

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These results indicate that resistin could lead to insulin resistance in mRNA and protein levels, but the resistin effect on insulin receptor in DNA level cannot be concluded. To detect whether insulin receptor promoter could be activated by exogenous resistin, luciferase reporter gene was fused downstream of human insulin receptor promoter and the enzymatic activity of luciferase was determined in the presence or absence of resistin.

MATERIALS AND METHODS

Reagents

Plasmid pSecTag2B, lipofectamine 2000, Opti-MEM, Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were bought from Invitrogen (Gibco, USA). Monoclonal rabbit anti-Myc antibody and fluorescein isothiocyanate (FITC) [or horseradish peroxidase (HRP)]-labeled goat anti-rabbit IgG (H+L) were from Beyotime® Institute of Biotechnology, China. All the restriction enzymes, VentR® DNA polymerase, DNA marker 2000 and T4 DNA ligase were purchased from New England Biolabs, UK. Primers were synthesized by Invitrogen. Luciferase assay system and pGL3-control were from Promega, USA. phINSRP-1, a plasmid containing a 1.8 kb promoter of human insulin receptor, was the generous gift from Dr. Graeme Bell (University of Chicago, IL, USA). Recombinant resistin protein was from Pepro Tech Asia (USA). Protein marker and enhanced chemiluminescence (ECL) were from Hou-Bio Tech. Co., Ltd., Hong Kong, China.

Cell culture

The normal human hepatic cells (L-02) were maintained by DMEM containing 10% FBS, and placed in the incubator under 5% CO_2 at 37 °C. The medium was freshened once every two days and the cells were passaged every three days.

Constructs

pSecTag2B-RETN was described previously (Qiao *et al*., 2007a). To develop pCMV-LUC, luciferase coding sequence was PCR amplified with forward (5′-GAT CGG ATC CGC CAC CAT GGA A-3′) and reverse (5′-GAC TCT CGA GCC ACG GCG ATC TTT-3′) primers from pGL3-control plasmid and digested with *Bam*HI/*Xho*I, followed by insertion into pSecTag2B vector. pCMV-LUC was restricted with *Spe*I/*Nhe*I to remove CMV promoter, and the construct was self-ligated to produce plasmid p0-LUC, in which the expression of reporter luciferase was not driven by any promoter. The promoter of insulin receptor (IRP) was amplified by PCR with forward (5′-GGG GGC TAG CGG CCA TTG CAC TCC A-3′) and reverse (5′-AAT TGG ATC CTG CGG GAG CGC GGG G-3′) primers taking phINSRP-1 as the template. The PCR products were digested with *Nhe*I/*Bam*HI and inserted into pCMV-LUC that was restricted with *Spe*I/*Bam*HI, yielding pIRP-LUC. All the constructs were confirmed by sequencing.

Transfection

L-02 cells were trypsinized and seeded with fresh medium without antibiotics at a density of 1.0×10^5 per well in a 24-well plate. After 24 h, cells were transfected with p0-LUC, pCMV-LUC and pIRP-LUC, respectively, according to the protocol provided by the manufacturer with some modifications. Three microlitres of lipofectamine 2000 was mixed with 47 μl of Opti-MEM and incubated for 5 min at room temperature. In another tube, 1 μg of luciferase-expressing plasmid was mixed with 50 μl of Opti-MEM. These two solutions were combined, mixed by tapping, and incubated for 20 min at room temperature. Cells were washed with prewarmed phosphate buffered saline (PBS), and then 100 μl Opti-MEM was added. The solution containing lipofectamine 2000 and plasmids was then added to the wells. After incubation for 4 h at 37 °C, growth medium was replaced with DMEM containing 10% FBS and cells were incubated further.

Immunofluorescence

L-02 cells were seeded in the 24-well plate containing a round cover slip overnight at 37 °C in serum-containing medium until they reached 50% confluence. After transfection with pSecTag2B-RETN, the cells were incubated for further 24 h. The cells were washed twice with PBS and fixed with 100% methanol for 5 min at room temperature, followed by blocking with PBS containing 10% defatted dry milk (blocking solution) for 20 min at room

temperature. Anti-Myc (C-term)-FITC antibody, diluted in the blocking solution (1:500), was added and incubated for 1 h in the dark at room temperature, and then imaged with confocal microscopy.

Western blot analysis

Whole cell lysate extracted from transfected L-02 cells (6-well) was separated by 15% SDS-polyacrylamide gel electrophoresis, electroblotted onto polyvinylidene difluoride (PVDF) membranes (Schleicher & Schuell, German), and immunodetected using rabbit anti-Myc antibody and goat anti-rabbit IgG-HRP conjugate. Bands were visualized with an ECL Western blot analysis system (Beyotime® Institute of Biotechnology, China).

Luciferase detection

L-02 cells were grown to 70% or 80% confluence on 24-well plates. Cells were transiently transfected by lipofectamine 2000 with 0.8 μg of pIRP-LUC, pCMV-LUC or p0-LUC, respectively. Recombinant resistin protein was added into wells 4 h post-transfection. Cell extracts were prepared 48 h after transfection, and the luciferase activity assay was performed with the reporter assay kits (Beyotime® Institute of Biotechnology, China). To correct for variability in transfection efficiency, each experiment was performed for at least 3 times, and each transfection was carried out in triplicate.

Statistical analysis

Group data are expressed as mean±*SEM*. Data were compared between experimental groups by two-way ANOVA. Differences between groups were further evaluated by Fisher-protected least squares differences. Differences were considered significant at a value of $P<0.05$.

RESULTS

Plasmid preparation

To detect the effect of resistin on the transcription of IRP, three luciferase-expressing vectors were constructed. In plasmid pIRP-LUC, the expression of luciferase reporter was controlled with IRP. When resistin was added to cells transfected with pIRP-LUC, resistin effect on IRP could be deduced by the enzyme activity of luciferase. pCMV-LUC will produce luciferase under CMV promoter, and there is no promoter to drive luciferase expression in p0-LUC.

pCMV-LUC produced a band corresponding to 1700 bp after digestion with *Bam*HI/*Xho*I, suggesting that luciferase coding sequence was inserted into pSecTag2B correctly. The bands about 1800 bp and 800 bp were released from pIRP-LUC and pCMV-LUC when restricted with *Bam*HI/*Bgl*II, respectively, indicating that CMV promoter was replaced with IRP. No band was found after the treatment of plasmid p0-LUC with *Bam*HI/*Bgl*II. All the plasmids were confirmed by sequencing and prepared with QIAGEN plasmid Miniprep Kit for transfection.

Effect of recombinant resistin on IRP transcription

In the above constructs luciferase was fused in frame with the Myc tag of pSecTag2B vector. To detect luciferase expression, total cell lysates were prepared and blotted with monoclonal rabbit anti-Myc antibody and subsequent HRP-labeled goat anti-rabbit IgG (H+L). Immune complexes were detected by enhanced chemiluminescence. A target band of about 61 kD was found from pIRP-LUC and pCMV-LUC-transfected cells when blotted with anti-Myc antibody (Fig.1), suggesting that luciferase was successfully expressed under the control of CMV or IRP promoter. The lysate from p0-LUC transfected cells did not produce bands.

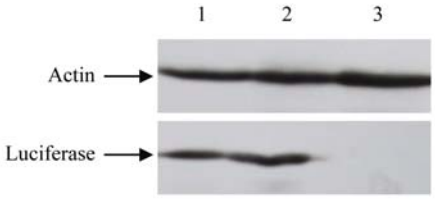

Fig.1 Western blot of luciferase expression

Cell extracts were prepared from pIRP-LUC, pCMV-LUC and p0-LUC transfected cells, respectively, and subjected for Western blot with anti-Myc antibody. A band of about 61 kD was found in transfected cells with either pIRP-LUC (Lane 1) or pCMV-LUC (Lane 2), suggesting that luciferase was successfully expressed under the control of CMV or IRP promoter. Luciferase was not expressed in the cells transfected with p0-LUC which has no promoter (Lane 3)

To detect the enzymatic activity of luciferase, luciferase-expressing plasmids were transfected and 4 h later recombinant resistin was added. Cell lysate was prepared 48 h post-transfection. Luciferase activity in cell lysates was determined with a luciferase assay kit and normalized to total cell protein as measured by bicinchoninic acid (BCA) assay kit. When treated with resistin protein, the enzymatic activity of pIRP-LUC transfected cells did not show significant difference compared to the untreated cells, suggesting that the transcription of IRP could not be activated by the added resistin (Fig.2). Unexpectedly, luciferase activity of pCMV-LUC transfected cells increased significantly in the presence of resistin when compared to that in the absence of resistin, the reason of which remains to be investigated. The cells transfected with p0-LUC showed much lower enzymatic activity, either in the presence or absence of resistin.

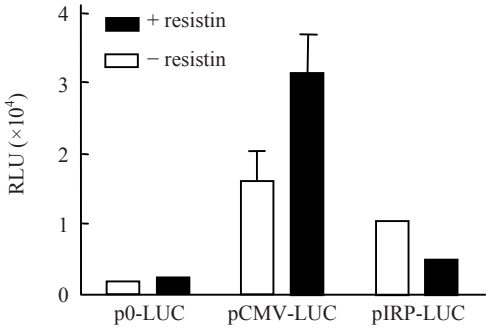

Fig.2 Enzymatic activity of luciferase

Luciferase-expressing plasmids driven by different promoters were transfected into L-02 cells, followed by the treatment of recombinant resistin or not. Cells were lysed and enzymatic activity of luciferase was detected 48 h post-transfection. Both pIRP-LUC and p0-LUC transfected cells did not show significant luciferase activity in the presence of resistin compared to that in the untreated cells (P>0.05). The treatment of pCMV-LUC with resistin significantly increased the luciferase activity (P<0.05). RLU: Relative light unit

Expressed resistin in the cytoplasm

Due to the finding that IRP transcription could not be activated by recombinant resistin, the endogenous resistin expressed with plasmid was stained with antibody against Myc tag that was in frame fused with the resistin coding sequence. To this end, resistin-expressing plasmid pSecTag2B-RETN was developed as described previously and transfected into L-02 cells. To detect the expression of resistin, cell lysate was prepared as above and the resistin protein was blotted with anti-Myc antibody in Western blot. The finding of a band corresponding to 13 kD indicates that resistin protein was expressed correctly (Fig.3). For immunostaining, cells were incubated with monoclonal rabbit anti-Myc antibody and FITC-labeled goat anti-rabbit IgG (H+L) in sequence 48 h post-transfection. Green fluorescence could be observed in the cytoplasm, but not in the nucleus (Fig.4). The results suggest that endogenous resistin may functionally locate in the cytoplasm but not enter the cell nucleus, corresponding to the above finding that IRP promoter could not be activated with recombinant resistin.

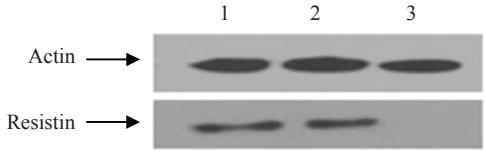

Fig.3 Western blot of resistin expression

L-02 cells were transfected with resistin-expressing plasmid and the cell lysate was extracted for detecting the expression of resistin with Western blot of anti-Myc antibody. Lanes 1 and 2: pSecTag2B-RETN transfected L-02 cells; Lane 3: Un-transfected L-02 cells

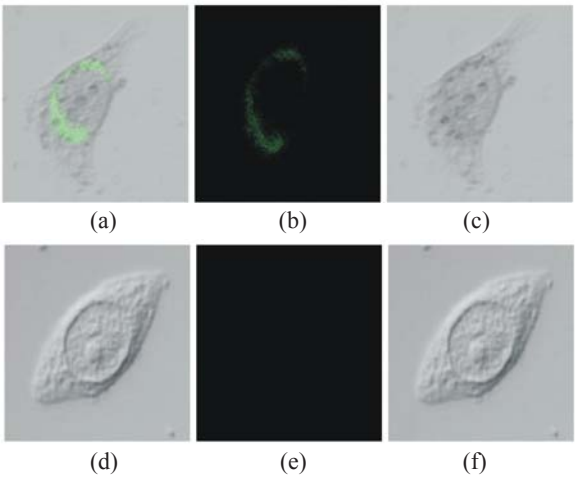

Fig.4 Expressed resistin locates in cytoplasm

pSecTag2B-RETN was transfected into L-02 cells and the expression was detected with immunostaining. (a), (b), (c) Green fluorescence was observed in the cytoplasm, but not in the nucleus (20× magnification), suggesting that expressed resistin locates in L-02 cytoplasm; (d), (e), (f) Green fluorescence was not detected in un-transfected L-02 cells (20× magnification). (a), (d) Merge channel; (b), (e) 488 nm channel; (c), (f) Transmission channel

DISCUSSION

Resistin belongs to the family of resistin-like molecules, and the resistin mRNA encodes a 114-amino acid polypeptide that contains a 20-amino acid signal sequence (Ukkola, 2002). Recent evidence has demonstrated that resistin is not expressed exclusively in adipose tissue, with at least one group demonstrating its expression in islet tissue and clonal β-cells (Minn *et al*., 2003). Resistin expression has also been identified in the liver and it has been suggested that resistin is rather in liver disease and may induce insulin resistance in humans (Yagmur *et al*., 2006).

In previous studies, we found elevated resistin level in serum with diabetes and coronary artery diseases (Qiao *et al*., 2007b), which was accordant with several other researches. For example, Tokuyama *et al*. (2007) reported that serum resistin concentrations were significantly higher in the insulin-resistant subgroup compared with the insulin-sensitive subgroup and correlated with insulin sensitivity; Al-Sari *et al*. (2007) found that resistin levels are higher in diabetic patients and positively correlated with body mass. In lean subjects, the resistin levels of serum have been reported to be approximately 5~15 ng/ml (Al-Daghri *et al*., 2005; Gerber *et al*., 2005), whereas in either T2D or obesity, the resistin levels increase to as high as 40 ng/ml (Fehmann and Heyn, 2002). These findings show that resistin might represent the long-sought link between obesity and insulin resistance.

Studies reported that resistin inhibits glucose uptake and impairs glucose tolerance, inducing insulin resistance via affecting insulin signal transduction pathway (Fan *et al*., 2007; Liu *et al*., 2008; Zhou *et al*., 2006). However, the relationship between insulin and insulin receptor was still poorly understood. The interaction of resistin and insulin receptor may be resulted from three factors as follows: (1) In DNA level, resistin down-regulates the promoter activity of insulin receptor; (2) In RNA level, resistin decreases mRNA expression of insulin receptor; (3) In protein level, resistin affects the activity of insulin receptor protein through direct or indirect interaction with insulin receptor protein.

In the present study, the treatment of pIRP-LUC transfected cells with 100 ng/ml resistin protein did not result in significant difference in enzymatic activity compared to the untreated cells, suggesting that the transcription of IRP could not be activated by the added resistin. However, Brown *et al*.(2007) reported that incubation with 40 ng/ml resistin significantly decreased insulin receptor mRNA and protein expression, but did not affect insulin secretion in mice. Liu *et al*.(2006) found that rat resistin concentrates in the cytoplasm around cell nucleus. To determine the location of human resistin in hepatocytes, we transfected L-02 cells with human resistin-expressing plasmid and found that the expressed resistin located in the cytoplasm in cell-staining, but not in the nucleus, confirming that human resistin does not down-regulate the transcription of insulin receptor in DNA level. It is not known whether rat resistin decreased the promoter activity of insulin receptor in mice. Species differences between mice and humans, or the cell specificity of hepatocytes and rodent pancreatic beta-cells may be other factors in the insulin resistance-based resistin. Further studies should be performed. Interestingly, luciferase activity of pCMV-LUC transfected cells increased significantly in the presence of resistin when compared to that in the absence of resistin protein, the reason of which remains to be investigated.

CONCLUSION

In summary, we determined the promoter activity of insulin receptor in the presence or absence of recombinant resistin by detecting the enzymatic activity of luciferase. The results indicate that resistin does not down-regulate the receptor promoter. Further studies of resistin function on insulin resistance will facilitate the development of therapeutic blockers in diabetic patients, and provide a new effective therapy for metabolic syndrome, T2D, and a series of cardiovascular diseases related to diabetes.

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