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Effects of over-expressing resistin on glucose and lipid metabolism in mice*

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Abstract: Resistin, a newly discovered peptide hormone mainly secreted by adipose tissues, is present at high levels in serum of obese mice and may be a potential link between obesity and insulin resistance in rodents. However, some studies of rat and mouse models have associated insulin resistance and obesity with decreased resistin expression. In humans, no relationship between resistin level and insulin resistance or adiposity was observed. This suggests that additional studies are necessary to determine the specific role of resistin in the regulation of energy metabolism and adipogenesis. In the present study, we investigated the effect of resistin in vivo on glucose and lipid metabolism by over-expressing resistin in mice by intramuscular injection of a recombinant eukaryotic expression vector pcDNA3.1-*Retn* encoding porcine resistin gene. After injection, serum resistin and serum glucose (GLU) levels were significantly increased in the pcDNA3.1-*Retn*-treated mice; there was an obvious difference in total cholesterol (TC) level between the experiment and the control groups on Day 30. In pcDNA3.1-*Retn*-treated mice, both free fatty acid (FFA) and high density lipoprotein (HDL) cholesterol levels were markedly lower than those of control, whereas HDL cholesterol and triglyceride (TG) levels did not differ between the two groups. Furthermore, lipase activity was expressly lower on Day 20. Our data suggest that resistin over-expressed in mice might be responsible for insulin resistance and parameters related to glucose and lipid metabolism were changed accordingly.

Key words: Resistin, Glucose, Lipid, Metabolism
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INTRODUCTION

The adipose tissue is an active endocrine organ directly involved in the control of metabolism, energy balance and reproductive function through a large number of secreted cytokines and hormones. These adipocytokines include leptin, interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), adiponectin and resistin. Resistin is a novel adipocytokine, together with RELM (resistin-like molecules)-α and RELM-β, belongs to the family of cysteine-rich RELMs (Steppan *et al*., 2001a). It was shown that the serum resistin levels are significantly increased in both genetic and diet-induced obese mice (Steppan *et al*., 2001b). Resistin may be a signaling molecule that is induced during adipocyte differentiation and specifically expressed and secreted by adipocyte (Kim *et al*., 2001; Gong *et al*., 2004). Moreover, obese mice given an anti-resistin antibody had increased insulin-stimulated glucose uptake, whereas the treatment of normal mice with recombinant resistin impaired insulin sensitivity (Steppan *et al*., 2001b). Therefore resistin could link obesity with insulin resistance and diabetes in mouse models. However, a subsequent study showed controversial findings on the role of resistin in obesity and insulin resistance rodent models (Banerjee *et al*., 2004). In humans, the physiological role is also questioned; because the resistin protein is almost undetectable in human adipocytes, macrophages and monocytes appear to be the princi-

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ple sources (Fain *et al*., 2003), and several studies have found no relationship between insulin resistance expression and insulin resistance or adiposity (Savage *et al*., 2001). It now appears that, in most obese patients, obesity is associated with a low-grade inflammation of white adipose tissue resulted from chronic activation of innate immune system, which can subsequently lead to insulin resistance, impaired glucose tolerance and even diabetes. During obesity, leptin, IL-6 and resistin are markedly increased (Bastard *et al*., 2006). Overall, the role of resistin in the lipid metabolism, glucose homeostasis, insulin resistance and relevant disease such as obese, and diabetes, has not been fully understood yet.

In this study, the recombinant plasmid pcDNA3.1-*Retn* coding for pig *Retn* gene was used to transfect cultured HeLa cells to examine whether the target gene could be expressed in mammalian cells. Then, pcDNA3.1-*Retn* plasmid was injected into mice. Serum concentrations of resistin, glucose (GLU), triglyceride (TG), free fatty acid (FFA), high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and total cholesterol (TC) were detected, and the effects of over-expressing resistin on glucose and lipid metabolism in mice were investigated.

MATERIALS AND METHODS

Animals

Six-week-old male KM mice were purchased from Guangdong Medical College (Zhanjiang, China) and housed in cages under controlled lighting conditions in a natural dark-light cycle. The mice were allowed free access to get diet and water.

Cell lines

HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated at 37 °C in a 5% CO_2 incubator.

Construction of pcDNA3.1-*Retn* expression vector and transfection

Using special primers F5′-CGG GAT CCA CCA TGA AGG CTT TGT CCT TG-3′ contained a *Bam*HI site and Kozak sequences, and R5′-GCC GGA ATT CCT ATG GAG TTC TCA ATC TAC-3′ contained an *Eco*RI site and stop code, the resistin gene fragment was amplified from pMD-*Retn* containing full length of porcine resistin gene. Eukaryotic expression vector of pcDNA3.1(+) (Invitrogen, USA) and PCR fragment were digested by restriction enzymes *Bam*HI and *Eco*RI. The digested vector and PCR fragment were ligated and used to transform *E. coli* Top10 competent cells. Recombinant clones were identified by *Bam*HI and *Eco*RI double digestion. Positive clones named pcDNA3.1-*Retn* were further confirmed by sequencing.

Cultured HeLa cells were transfected with recombinant pcDNA3.1-*Retn* and pcDNA3.1(+) respectively. Transfection was performed according to the instructions of Lipofectamine™ 2000 reagent kit (Gibco, USA). Cells were cultured in DMEM containing 10% FBS and 800 μg/ml G418. The resistant cell clones were harvested 2 weeks after transfection.

Detection of recombinant resistin in HeLa cells

Total RNA was extracted from transfected and control cells using Trizol® reagent (Gibco, USA) according to the manufacturer's protocol. RNA was reverse transcribed at 37 °C for 60 min, and the products were used as the template for PCR reaction. The amplified products were detected on 1.5% agarose gel electrophoresis.

Cells were lysed in a buffer containing 20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, and 2 mmol/L phenylmethylsulfonyl fluoride for 30 min in ice-bath. The proteins of cell lysis were separated by 15% SDS-PAGE and transferred to nitrocellulose (NC) membrane (Millipore, USA). The membrane was blocked for 1.5 h using 5% nonfat dried milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T), and incubated at 4 °C overnight in the presence of rabbit anti-procine resistin serum. The membrane was then incubated with a goat anti-rabbit IgG labelled with horseradish peroxidase (HRP) for 1 h. The specific recombinant resistin band was displayed with 10 ml 0.01 mol/L Tris-Cl (pH 7.6) containing 6 mg 3,3′-diaminobenzidine (DAB), 0.03% $CoCl_2$ and 10 μl 30% H_2O_2.

Animal experiment

The plasmids pcDNA3.1-*Retn* and pcDNA3.1(+)

were prepared with a regular method. Purified plasmid DNA was quantified by spectrophotometry at 260 nm and the final concentration was adjusted to 1 μg/μl in phosphate-buffered solution.

Sixty 6-week-old male KM mice were randomly divided into 2 groups. Each mouse in the control group received intramuscular injection with 100 μg pcDNA3.1(+) and each mouse in the experiment group was injected with 100 μg pcDNA3.1-*Retn* for 3 times and the interval of each administration was 10 d. In order to improve the absorption of the plasmid, all animals were injected with 0.25% lidocaine in the quadriceps of each hind leg one day before injection. Ten days after each injection, blood samples were obtained from the eyeball extirpation of the mice, plasma samples were immediately prepared by centrifugation at 1500 r/min for 15 min at 4 °C and stored at −80 °C

Biochemical analysis

The resistin concentrations in mice serum were detected by the quantitative sandwich enzyme immunoassay technique (mouse resistin ELISA kit, ADL Co., USA).

GLU, FFA, TG, HDL cholesterol, LDL cholesterol and TC were determined at 10, 20 and 30 d after injection, using commercial kit (Huili Co., Changchun, China) according to manufacturer's instructions.

Statistical analysis and ethical considerations

Values are presented as mean±*SEM*. Results were analyzed with software SPSS 12.0 using student's *t*-test or one-way ANOVA. $P<0.05$ denoted the presence of a statistically significant difference. The experiment protocol was approved by the Ethics Review Committee for Animal Experimentation of Guangdong Ocean University, China.

RESULTS

Detection of recombinant resistin expressed in HeLa cells

A 330 bp fragment was amplified from plasmid pMD-*Retn* by PCR. Restriction endonuclease digestion and DNA sequencing results showed that recombinant plasmid pcDNA3.1-*Retn* was constructed successfully and the open reading frame (ORF) was corrected (Fig.1). RT-PCR detection showed that a specific 330 bp fragment could be amplified from total RNA template extracted from positive HeLa cell clones transfected with recombinant plasmid pcDNA3.1-*Retn* (Fig.2), and the control cell clones transfected with pcDNA3.1(+) and untransfected HeLa cells were negative. Western blot analysis of the HeLa cell lysis indicated that there was a specific anti-resistin response band on NC membrane and no band was found in the extracts from HeLa cells transfected with pcDNA3.1(+) (Fig.3). The results above suggested that resistin gene could be correctly expressed in the transfected HeLa cells.

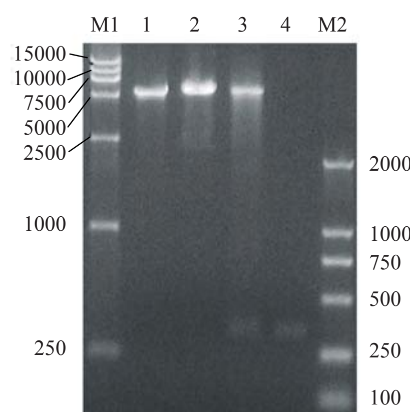

Fig.1 Restriction analysis of pcDNA3.1-*Retn* recombinant plasmid

M1: DL 15000 DNA marker; M2: DL 2000 DNA maker; 1: Plasmid pcDNA3.1(+) digested with *Bam*HI and *Eco*RI; 2: Recombinant plasmid pcDNA3.1-*Retn* digested with *Bam*HI; 3: Recombinant plasmid pcDNA3.1-*Retn* digested with *Bam*HI and *Eco*RI; 4: PCR product of *Retn* fragment

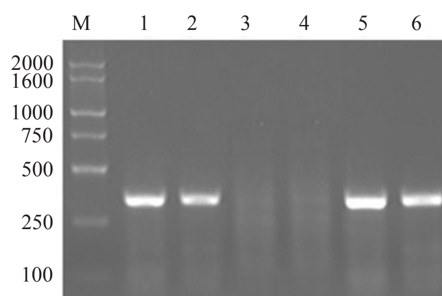

Fig.2 RT-PCR analysis of *Retn* mRNA stable expression in HeLa cells

M: DL 2000 DNA marker; 1, 2, 5 and 6: RT-PCR products of different HeLa cell clones transfected with pcDNA3.1-*Retn*; 3: RT-PCR product of HeLa cells transfected with pcDNA3.1(+); 4: RT-PCR product of untransfected HeLa cells

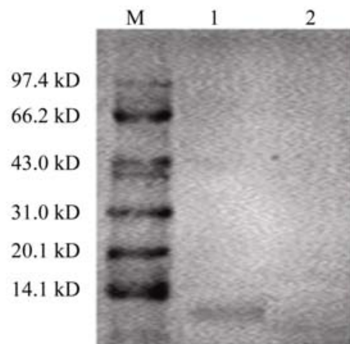

Fig.3 Western blot analysis of pcDNA3.1-*Retn* stable expression in HeLa cells
M: Prestained protein molecular marker; 1: HeLa cells transfected with pcDNA3.1-*Retn*; 2: HeLa cells transfected pcDNA3.1(+)

Change of serum resistin level

At different times after injection, the experiment group showed a significantly higher resistin level than that of control group. This difference developed with time was more and more obvious (Fig.4a). On Day 30, the concentration of serum resistin in the experiment group was 17.12 μg/L, but in the control group was 12.14 μg/L.

Change of serum GLU level

The concentrations of serum GLU in pcDNA3.1-*Retn*-treated mice were significantly higher than those in control group on Day 10 (pcDNA3.1-*Retn*-treated mice, (4.7±0.43) mmol/L; pcDNA3.1(+)-treated mice, (3.4±0.23) mmol/L) and Day 30 (pcDNA3.1-*Retn*-

■ pcDNA3.1-*Retn* (experiment group)
▥ pcDNA3.1(+) (control group)

Fig.4 The levels of serum resistin (a), GLU (b), FFA (c), TC (d), TG (e), HDL (f) and LDL (g) at different times after injection
n=8; $^{*}P<0.05$; $^{**}P<0.01$

treated mice, (5.3±0.39) mmol/L; pcDNA3.1(+)-treated mice, (3.3±0.32) mmol/L). On Day 20, serum GLU concentration increased by 25.2% in the experiment group (Fig.4b), and there was no significant difference between the two groups (pcDNA3.1-*Retn*-treated mice, (6.09±0.71) mmol/L; pcDNA3.1(+)-treated mice, (4.87±0.54) mmol/L).

Changes of serum FFA, TC and TG levels

The serum FFA concentration was significantly lower in pcDNA3.1-*Retn*-treated mice than that of control group on Day 20 (pcDNA3.1-*Retn*-treated mice, (1.5±0.16) μmol/L; pcDNA3.1(+)-treated mice, (2.49±0.18) μmol/L) and Day 30 (pcDNA3.1-*Retn*-treated mice, (1.8±2.70) μmol/L; pcDNA3.1(+)-treated mice, (2.64±0.23) μmol/L). The concentration of serum FFA also decreased in the pcDNA3.1-*Retn*-treated mice on Day 10 ((1.4±0.17) μmol/L) (Fig.4c). Serum TC concentration was lower in mice that received pcDNA3.1-*Retn* compared with control group given pcDNA3.1(+) during the experimental period, but significant differences were only evident on Day 30 (pcDNA3.1-*Retn*-treated mice, (1.05±0.13) mmol/L; pcDNA3.1(+)-treated mice, (1.50±0.09) mmol/L) (Fig.4d). Surprisingly, the mice in the experiment group had slightly higher TG level than that of control group during the experimental period ($P>0.05$) (Fig.4e).

Changes of serum HDL and LDL levels

After injection, the experiment group showed a significantly lower HDL cholesterol level than that of control group on Day 10 (pcDNA3.1-*Retn*-treated mice, (0.37±0.01) mmol/L; pcDNA3.1(+)-treated mice, (0.50±0.03) mmol/L), Day 20 (pcDNA3.1-*Retn*-treated mice, (0.37±0.01) mmol/L; pcDNA3.1(+)-treated mice, (0.54±0.03) mmol/L) and Day 30 (pcDNA3.1-*Retn*-treated mice, (0.32±0.02) mmol/L; pcDNA3.1(+)-treated mice, (0.41±0.03) mmol/L) (Fig.4f). The serum LDL cholesterol level of the experiment group was slightly lower than that of control group, and there was no significant difference between the two groups (Fig.4g).

DISCUSSION

Resistin, a putative adipocyte-derived signalling polypeptide, was originally implicated as a factor linking obesity and diabetes by impairing insulin sensitivity and glucose tolerance in mice. Resistin circulated at increased levels in murine models of obesity and insulin resistance and was negatively regulated by thiazolidenediones (Steppan *et al*., 2001b; Steppan and Lazar, 2002). Resistin has been shown to impair insulin signaling and/or glucose metabolism in liver (Banerjee *et al*., 2004; Rajala *et al*., 2003), skeletal muscle (Pravenec *et al*., 2003; Satoh *et al*., 2004) and adipose tissues (Satoh *et al*., 2004; Steppan *et al*., 2005). In L6 rat skeletal muscle cells in vitro, whether long- or short-term resistin incubation, impair glycogen synthesis by reducing the rate of glucose-6-phosphate both in the absence and presence of insulin (Palanivel *et al*., 2006; Niederwanger *et al*., 2007). The results above suggest that resistin takes part in the regulation of glucose and energy homeostasis. The relationship between resistin and lipid metabolism also has been observed, but the results still remained inconsistent.

In the present study, the recombinant plasmid pcDNA3.1-*Retn* expressing porcine resistin gene, was used to transfect HeLa cells, and the recombinant resistin protein was detected by SDS-PAGE and Western blot. On both of PAGE gel and NC membrane, a specific 12 kDa protein band was detected. It was confirmed that resistin could be expressed in mammalian cells.

Next, the effects of over-expressing resistin on glucose and lipid metabolism in mice were observed. During the experimental period, the experiment group showed a significantly higher resistin level than that of the control group, suggesting that resistin was over-expressed in pcDNA3.1-*Retn* treated mice. The results indicated that the pcDNA3.1-*Retn* treated mice showed higher GLU and TG concentrations than those of the control group during the experimental period. These findings are consistent with the previous reports: the serum GLU level acutely reached the peak as the mice received recombinant resistin by intraperitoneal injection (Steppan *et al*., 2001a), and the glucose tolerance and insulin sensitivity of the mice implanted with cells that over-express resistin were impaired simultaneously (Kitagawa *et al*., 2004). The reasonable explanation is that over-expressing resistin leads to insulin resistance, and the elevation of serum GLU level may contribute to the reduced amount of glucose transported into certain target cells to be oxidated (Palanivel *et al*., 2006; Niederwanger *et al*., 2007). However, in other studies, the glucose metabolism was not affected by over-expressing resistin protein in mice body using intravenous administration of recombinant adenovirus encoding mouse resistin gene (Satoh *et al*., 2004; Sato *et al*., 2005).

FFA, due to the interfering role of insulin signaling, is considered as a prominent sign of insulin resistance of skeletal muscle, liver and adipose tissue (Palanivel and Sweeney, 2005; Hegarty *et al*., 2002). The serum resistin level was markedly elevated in mice fed on high-fat (containing 45% of fat) for four weeks, and the mice became insulin resistant and obese (Steppan *et al*., 2001a). Similarly, the mice fed on high-fat diet also showed higher resistin expression, insulin resistant and impaired glucose intolerance, and moreover the circulating FFA was significantly decreased (Chen and Nyomba, 2003) and seemed to be ambivalent to the insulin resistant status. In the present study, plasma FFA concentration of the experiment group was significantly lower than that of the control group, which was in accordance with the previous investigation (Chen and Nyomba, 2003). We presumed that, in the process of insulin resistance, the glucose uptake of skeletal muscle or other tissues decreased, and as the compensation mechanism, more FFA was used to oxidation to maintain energy homeostasis, and the efficiency of FFA utilization and clearance was subsequently increased. As a result of insulin resistance and FFA flux to the liver to be oxidized, TG synthesis was stimulated and the plasma TG concentration increased accordingly. However, there was no significant difference between the pcDNA3.1-*Retn* treated group and the control group. After injection the serum TC level did not differ between the two groups on Day 10 or 20. However, the TC concentration was significantly lower in pcDNA3.1-*Retn* treated mice than that of control group on Day 30 after injection. It is clear that over-expression of resistin may impair the regulation of storage and release of energy by adipose, and the metabolisms of GLU, FFA and TG are changed profoundly.

Over-expressing resistin in mice can affect lipid metabolism independent of obesity or a high fat diet. In fact, both LDL and HDL are important delivery manners of FFA, TG and cholesterol; as LDL and HDL secretion increased, the plasma FFA flux to the liver to be oxidated was accordingly increased (Dixon and Ginsberg, 1993; Lewis, 1999). By contrast, we found no significant difference in serum LDL values between the two groups, whereas Sato *et al*.(2005) reported that mice treated with resistin adenovirus showed a higher plasma LDL and cholesterol levels. This result may be partially due to the difference in the animal breeds, and/or genetic background, and dietary conditions. Although we could not detect any difference in serum LDL level between the two groups, we still observed the serum HDL level significantly decreased in the pcDNA3.1-*Retn* treated mice. These findings are partially inconsistent with the previous report: when adenovirus-mediated over-expression of resistin was achieved in normal chow-fed mice, plasma TG levels increased, and this was associated with increased secretion of TG from the liver, and LDL cholesterol also increased while HDL cholesterol fell in the mice (Sato *et al*., 2005).

The consensus RELMs are consisted of 105~114 amino acids with unique cysteine residues pattern: C-X11-C-X8-C-X-C-X3-C-X10-C-X-C-X-C-X9-CC-X3-6 (Steppan *et al*., 2001b), and this unique pattern likely contributes to folding, multimerization and biological function of the RELMs. The previous reports have not concerned the diverse roles and tissue specificities, and it is unclear that whether porcine resistin plays a similar role as murine resistin. It has been reported that the porcine resistin gene was composed of four exons and had exactly the same exon structure as the human resistin gene, and the deduced amino acid sequence showed 75.2% identity to the human resistin (Dai *et al*., 2006). In the present study, we found that the homology of amino acids sequence of the matured resistin peptides between the rodent and porcine is about 62.2%~78.6%, and moreover, the amino acid constitution adjacent to the cysteine residues is almost the same, suggesting that resistin originating from porcine and rodent plays an analogous role in regulation of glucose and lipid metabolism. However, additional work will be needed to understand the tissue-specific functions and mechanisms of each member of RELMs.

In conclusion, we have demonstrated that over-expressing resistin in mice led to insulin resistance and dysregulation of glucose, lipid and energy metabolism. As a result of insulin resistance in mice, the serum GLU and TG levels increased, and the serum FFA, HDL cholesterol and TC concentrations decreased significantly at different stages of the experiment. It has been reported that glucose and lipid metabolism can be influenced by several adipose tissue-derived factors such as FFA (Gao *et al*., 2004), TNF-α (Borst *et al*., 2004), leptin (Friedman, 2000) and adiponectin (Yamauchi *et al*., 2003). More recently, several studies suggested that insulin resistance is related to pro-inflammatory condition that promotes the production of inflammatory factors such as TNF-α and IL-6 (Bastard *et al*., 2006). Obviously, the mechanisms of the regulations of glucose, lipid and energy metabolism are complex indeed and incompletely understood; therefore, further studies are necessary to elucidate the physiological roles in insulin resistance and energy homeostasis.

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