# Resistin overexpression impaired glucose tolerance in hepatocytes

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**ABSTRACT. Resistin is a 12.5-kDa cysteine-rich protein secreted from adipose tissue and is an important factor linking obesity with insulin resistance. Here, we investigated the effect of resistin on glucose tolerance in adult human hepatocytes (L-02 cells). In this study, resistin cDNA was transfected into L-02 cells, and glucose concentration and glucokinase activity were determined subsequently. The data indicated resistin impaired, insulin-stimulated glucose utilization, which implied liver was a target tissue of resistin. To understand its molecular mechanism, mRNA levels of key genes in glucose metabolism and insulin signaling pathway were analyzed. The results demonstrated resistin-stimulated expression of glucose-6-phosphatase (G6Pase), sterol regulatory element-binding protein 1c (SREBP1c) and suppressor of cytokine signaling 3 (SOCS-3), repressed expression of peroxisome proliferator-activated receptor γ (PPARγ) as well as insulin receptor substrate 2 (IRS-2). Given that glucokinase (GK) activity and glucose transporter 2 (GLUT2) expression were not altered, we presumed that resistin did not effect them. Moreover, resistin lowered mRNA levels of IRS-2 while stimulating SOCS-3 expression, which suggests it impairs glucose tolerance by blocking the insulin signal transduction pathway.**

**Key words:** glucose tolerance, insulin resistance, resistin, rosiglitazone

Obesity is a major risk factor for insulin resistance and type 2 diabetes mellitus. Although this association is well established, the mechanisms have not been elucidated. Accumulating evidence, both *in vitro* and *in vivo*, has demonstrated that adipocytokines such as TNFα, leptin, adiponectin and resistin are involved in the regulation of insulin activity. Resistin, also known as adipocyte-secreted factor and Fizz3 [1, 2], has been recently postulated to be a link between obesity and insulin resistance [3].

Resistin is composed of 114 amino acids when synthesized, shedding 20 amino acids when mature [4], and was originally considered to be involved in insulin resistance. *In vivo* and *in vitro* studies have demonstrated that high concentrations of resistin impair insulin sensitivity and cause insulin resistance. In diet-induced obese mice, immunoneutralization of resistin led to a 20% drop in blood glucose, and improved insulin sensitivity. In contrast, recombinant resistin impairs insulin-stimulated glucose uptake [5]. Moreover, overexpression of resistin leads to inhibition of adipocyte differentiation [2], which implies that resistin is an inhibitor in adipogenesis. Recent works also indicated that resistin is closely linked to liver cirrhosis [6] and hepatic insulin resistance [7].

As we know, the liver, a key metabolic organ, is bifunctional in glucose metabolism, namely glucose utilization and production. It plays a crucial role in regulating energy balance, and has a close relationship with insulin sensitivity and glucose tolerance. As a result, hepatic insulin resistance is also presumed to be chiefly responsible for the development of type 2 diabetes [8]. Current *in vivo* studies indicate that infusion of resistin dramatically increases glucose production and impairs insulin action in the liver, whereas its specific anti-sense oligodeoxynucleotide reverses these effects [7]. The results suggest that resistin directly impairs glucose uptake and insulin action in liver.

To investigate the biological effect of resistin on hepatic glucose metabolism, we cultured human L-02 cells with transfected resistin and examined the effect of resistin on insulin-stimulated glucose utilization and on the expression of relevant genes.

## MATERIALS AND METHODS

### *Materials*

TRIzol was purchased from Sangon Co., Ltd. (Shanghai, China); Mammalian Cell Protein Extraction kit from Shenergy Biocolor BioScieTechnology Co., Ltd. (Shanghai, China); Glucose Assay Kit from Shanghai Shenergydiasys Diagnostic Technology Co. Ltd. (Shanghai, China); Dulbecco's modified Eagle's medium (DMEM) and

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Lipofectamine™ 2000 from Invitrogen/GIBCO; all restriction endonucleases were from TaKaRa Co. (Dalian, China).

### *Plasmid construction*

Full-length resistin (accession No: NM_022984) was obtained using RT-PCR. The PCR products that contained *Eco*RI-*Bam*HI restriction sites were subcloned into the pIRES2-EGFP vector, in which there was an internal ribosome entry site (IRES) between the MCS and EGFP genes. The plasmid was named pI-RE. The resistin and EGFP genes are translated from a single bicistronic mRNA, but develop two separate proteins.

### *Plasmid extraction*

Plasmids were reproduced in *Escherichia coli* DH5α and were extracted as described previously [9]. Briefly, the whole process included two steps: first step, lysis- plasmid DNA was isolated from 100 mL bacterial culture by treatment with alkali and SDS; second step, purification- plasmid DNA was purified by precipitation with polyethylene glycol 8000. The criterion for plasmid DNA purity was OD260/OD280 ≥ 1.7. The concentration of plasmid DNA was diluted to 1 μg/μL.

### *Cell culture and treatment*

The L-02 cell line was derived from adult human liver [10], grown in DMEM supplemented with 10% fetal bovine serum at 5% $CO_2$ and 37°C. For the relevant experiments, the density of cells was about 5 x $10^5$ cells/well in 24-well culture plates for RNA extraction; or 5 x $10^6$ cells/dish in 60-mm Petri dishes for metabolite concentration assay. There were four cell groups in the experiments, namely basal, IT, RT and RRT. The basal group was cultured with basal medium- DMEM supplemented with 10% fetal bovine serum. Other groups were treated with 10 nM insulin. The IT group (insulin treatment) was transfected by pIRES2-EGFP. In addition, the RT group (resistin treatment) was transfected by pI-RE; the RRT group (rosiglitazone and resistin treatment) was also transfected by pI-RE and given 10 μM rosiglitazone which is a type of TZD. All treatments are listed in *table 1*.

### *Transient transfection in L-02 cells*

The expression vectors were transiently transfected into L-02 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, the vectors were firstly transfected into cells in a serum-free medium and changed to DMEM with 10% FBS six hours later. The cells were collected 36 hours after transfection to isolate their RNAs.

### *RNA isolation*

Total RNAs were isolated from L-02 cells using TRIzol. All of the RNA samples were treated with Dnase I to digest the genomic DNA and stored at -80°C before further processing.

### *Semi-quantitative RT-PCR*

Semi-quantitative RT-PCR with ribosomal protein L19 (RPL19) as an internal control was performed to determine the mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ), insulin receptor substrate 2 (IRS-2), glucose transporter 2 (GLUT2), sterol regulatory element-binding protein 1c (SREBP1c), suppressor of cytokine signaling 3 (SOCS-3) and glucose-6-phosphatase (G6Pase) in L-02 cells. All primers are listed in *table 2*. Preliminary experiments were carried out with various PCR cycles to determine the linear range of amplification for all genes.

### *Protein isolation and concentration assay*

Proteins were isolated using the Mammalian Cell Protein Extraction kit. Protein concentrations were determined using the Bradford assay [11].

### *Glucose concentration assay*

The glucose concentration in medium was assayed using the Glucose Assay Kit. Absorbance was assayed at 340 nm using Beckman Coulter DU 800 UV/Visible spectrophotometer. All sample concentrations were normalized by each protein amount. The reduction in glucose concentration was calculated by subtracting the glucose concentration at the end from the starting glucose concentration.

### *Glucokinase (GK) assay*

Enzymatic activity was assayed as described previously [12], using NAD as coenzyme and glucose-6-phosphate dehydrogenase as the coupling enzyme. The assay buffer contained 100 mM triethanolamine hydrochloride (Tris-HCL, pH 7.8), 5 mM $MgCl_2$, 5 mM ATP, 150 mM KCl, 2 mM dithiothreitol, 0.2% bovine serum albumin, 1 mM NAD, and 1 unit/ml of G6PDH. Correction for low hexokinase activity was applied by subtracting the activity measured at 0.5 mM glucose from the activity measured at 100 mM glucose. Absorbance was measured at 340 nm

**Table 1**
Description of cell treatment

| Group | Basal | IT | RT | RRT |
|---|---|---|---|---|
| **Agent** | | | | |
| Basal medium | + | + | + | + |
| Insulin (10 nM) | | + | + | + |
| pIRES2-EGFP | + | + | | |
| pI-RE | | | + | + |
| Rosiglitazone (10 μM) | | | | + |

**Table 2**
Primers used for semi-quantitative RT-PCR

| Gene name Size (bp) | Size (bp) | Forward and Reverse primer (5′-3′) | Accession number |
|---|---|---|---|
| PPARγ | 195 | F: TCTCCAGTGATATCGACCAGC | **BT007281** |
| | | R: TTTTATCTTCTCCCATCATTAAGG | |
| IRS-2 | 383 | F: CACCTCCCCACGACAGTTGC | **NM_003749** |
| | | R: GGTGGGACAAGAAGTCAATGCTG | |
| GLUT2 | 398 | F: TTTTCAGACGGCTGGTATCAGC | **J03810** |
| | | R: CACAGAAGTCCGCAATGTACTGG | |
| SREBP1c | 248 | F: CACCGTTTCTTCGTGGATGG | **BC057388** |
| | | R: CCCGCAGCATCAGAACAGC | |
| SOCS-3 | 364 | F: TTCAGCTCCAAGAGCGAGTACC | **NM_003955** |
| | | R: CGGAGGAGGGTTCAGTAGGTG | |
| G6Pase | 244 | F: CGACCTACAGATTTCGGTGCTTG | **NM_000151** |
| | | R: AGATAAAATCCGATGGCGAAGC | |
| RPL19 | 419 | F: GGCTCGCCTCTAGTGTCCTC | **NM_000981** |
| | | R: GTCTGCCTTCAGCTTGTGG | |

using Beckman Coulter DU 800 UV/Visible spectrophotometer. Enzymatic activity was normalized by the amount of protein.

### *Statistical analysis*

Results are expressed as mean ± S.E. of 3 independent experiments in triplicate. The comparison was carried out using Student's *t* test. The significance level chosen was $p < 0.05$.

## RESULTS

### *Resistin expressed in L-02 cells by transient transfection*

Twenty four hours after transfection, resistin expression was measured by fluorescence photography and semi-quantitative RT-PCR. Because both the resistin and EGFP genes were in the same RNA, and EGFP was downstream of resistin, the fluorescence of EGFP represented resisin expression *(figure 1A)*. The results from semi-quantitative RT-PCR indicated resistin expression more directly *(figure 1B)*.

### *Resistin reduced insulin-stimulated glucose consumption, but had little effect on glucokinase activity*

To investigate whether resistin is correlative with insulin action, glucose concentration was determined at 0 h, 18 h and 36 h, respectively. Glucokinase is one of the main hexokinases in the liver; it plays a key role in regulating glucose phosphatization. It was also measured at 0 h, 18 h and 36 h, respectively. Six hours after transfection, the medium was changed to DMEM with 10% FBS, and this time-point was marked as 0 h to assay relative indexes. The results showed that resistin suppressed insulin-stimulated glucose consumption markedly (43% reduction, $p < 0.01$, 18 h; 31% reduction, $p < 0.01$, 36 h) *(figure 2A)*, but had no effect on GK activity *(figure 2B)*, which indicated that resistin impaired glucose utilization while glucokinase did not play a key role in this process.

### *Rosiglitazone reversed the influence of resistin, and enhanced glucose consumption and glucokinase activity*

Rosiglitazone is able to improve insulin sensitivity and enhance glucose tolerance; as a result, its relationship with resistin was examined in this study. Glucose concentration

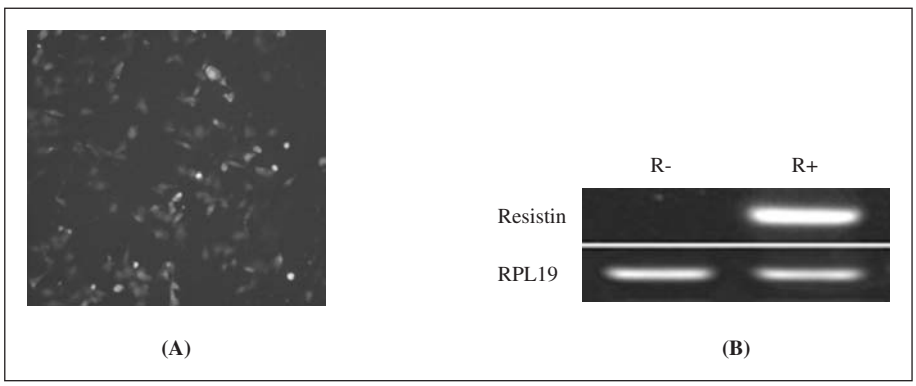


**Figure 1**
pI-RE was transfected into L-02 cells. Twenty four hours after transfection, fluorescence (**A**) and resistin mRNA levels (**B**) were determined. Results indicated that resistin was overexpressed in hepatocytes. R- represents the control group that did not transfect with pI-RE, R+ represents the group that transfected with pI-RE.

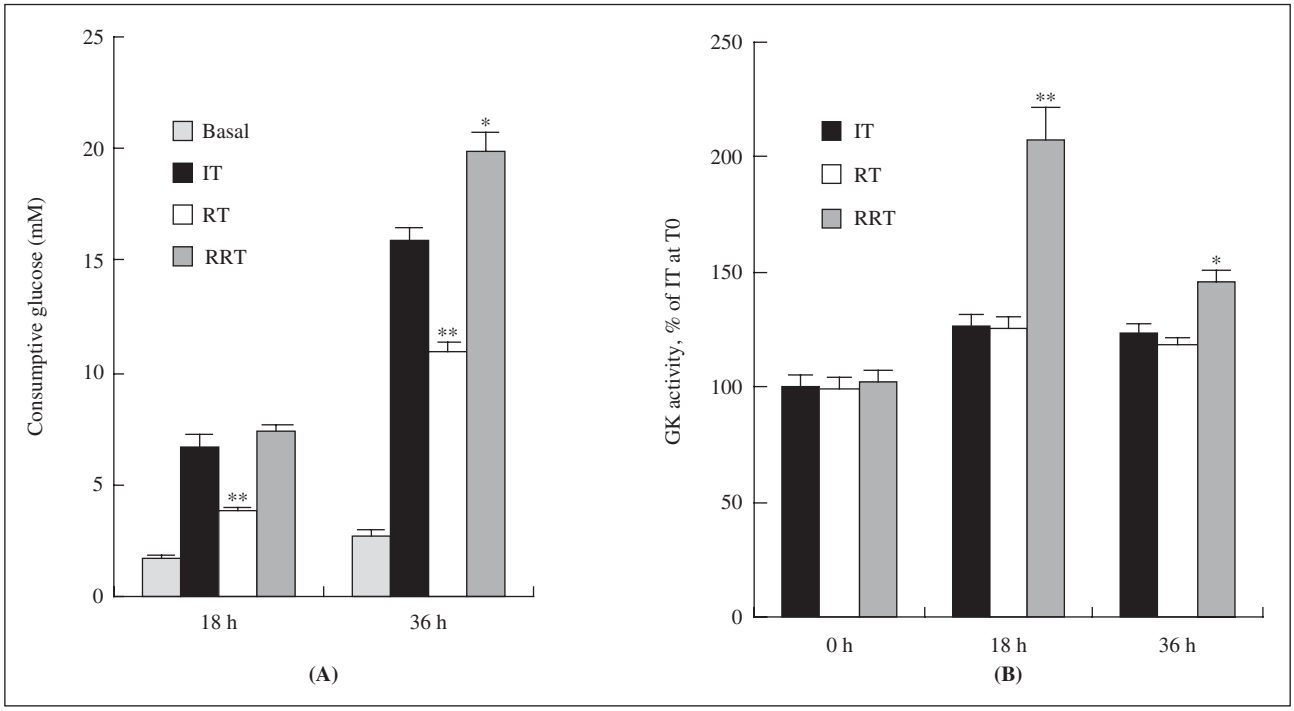


**Figure 2**

Effects of resistin on glucose utilization and glucokinase activity.
After plating, L-02 cells were cultured for 36 h as described in materials and methods. After 0, 18, and 36 h, the glucose concentration in medium (**A**) and GK activity (**B**) were determined. Data were normalized by the amount of protein and were given as mean ± S.E. of 3 independent experiments in triplicate. **A**) * ($p < 0.05$), ** ($p < 0.01$) indicated that glucose consumption in the treatment group was significant greater or lower than in the control group (IT group). **B**) * ($p < 0.05$), ** ($p < 0.01$) indicated that GK activity in the treatment group was significant greater or lower than in the control group (IT group).

and glucokinase activity were assayed at 0 h, 18 h and 36 h, respectively. Six hours after transfection, the medium was changed to DMEM with 10% FBS and 10 μM rosiglitazone, and this time-point was marked as 0 h to assay relative targets. The data indicated glucose consumption (10% induction, 18 h; 25% induction, $p < 0.05$, 36 h) and GK activity (63% induction, $p < 0.01$, 18 h; 19% induction, $p < 0.05$, 36 h) were enhanced with rosiglitazone treatment *(figure 2)*, which suggested that rosiglitazone was able to stimulate glucose utilization that was suppressed by resistin. Interestingly, glucose consumption of the rosiglitazone treatment group was greater than that of the control group, which implies that treatment with rosiglitazone causes cells to consume more glucose.

### *Effect of resistin on the glucose metabolic components*

It is well recognized that glucose metabolism is modulated by GLUT and G6Pase. GLUT2 is the primary glucose transporter isoform in the liver regulating glucose transport. G6Pase is an important determinant of the hepatic glucose fluxes regulated by insulin. Effects of resistin were measured to further understand the mechanism of how it impairs insulin-stimulated glucose consumption. All cells were cultured for 36 h before cell RNAs were isolated. The results from semi-quantitative RT-PCR *(figure 3A)* demonstrated that overexpression of resistin stimulated G6Pase expression (2.4-fold induction, $p < 0.01$), but had little effect on GLUT2 expression *(figure 3B)*.

### *Effect of resistin on the insulin-signaling components*

As previous research has revealed, PPARs and SREBPs are well-characterized transcription factors. Previously, PPARγ was thought to be the main isoform in adipocytes, however, recent discoveries have indicated that it might also mediate lipid metabolism and energy homeostasis when its expression in the liver is altered. Research has also shown that TZDs improved insulin sensitivity mainly by upregulating PPAR expression, which suggested that PPAR was closely linked to insulin sensitivity. SREBP1c is crucial for the regulation of the lipogenic gene and from recent studies, is considered to be relevant to insulin action. Moreover, IRS-2 and SOCS-3 are crucial factors with opposing functions in the insulin signaling pathway. IRS-2 expression improves insulin sensitivity, but SOCS-3 is known as an insulin signaling inhibitor. Obviously, the expression variation of these genes would influence the action of insulin. To explore the relationship between resistin and insulin action, the mRNA levels of the above genes were measured. All cells were cultured for 36 h before cell RNAs were isolated. The results from semi-quantitative RT-PCR *(figure 4*A*)* demonstrated that resistin stimulated expression of SOCS-3 (2.5-fold induction, $p < 0.01$) and SREBP1c (33% induction, $p < 0.05$),

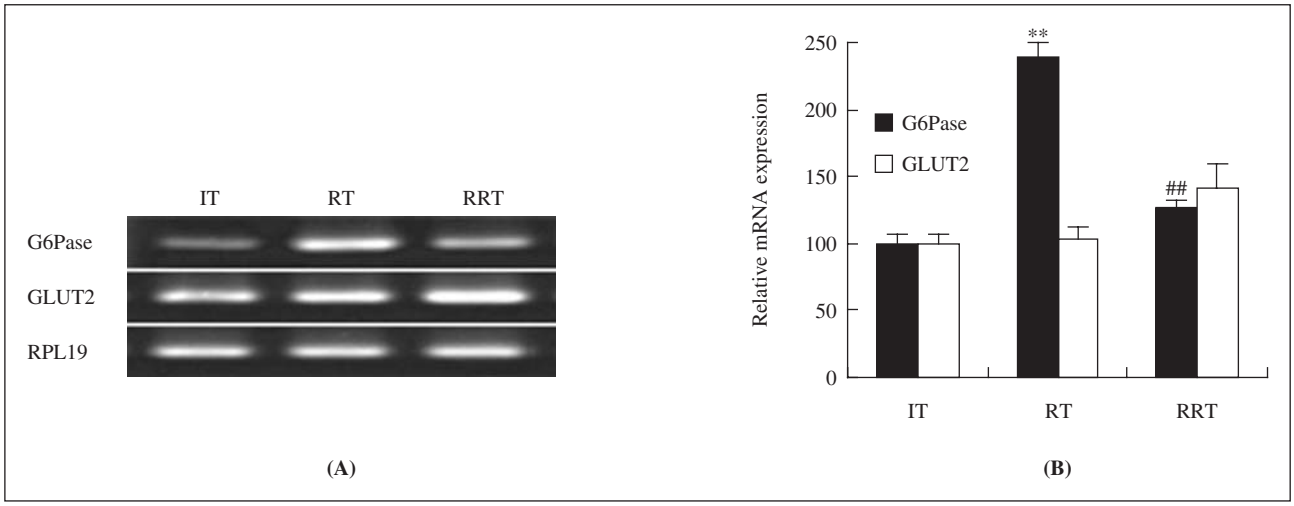


**Figure 3**

Effects of resistin on the glucose metabolic components.
After plating, hepatocytes were cultured for 36 h as described in materials and methods. After 36 h, total RNA was extracted and analyzed for the expression of G6Pase and GLUT2 (**A**). The quantification of blots for expression of these genes, obtained in 3 independent experiments in triplicate, was shown (**B**). RPL19 was used as an internal control to normalize the expression levels of these genes. * ($p < 0.05$), ** ($p < 0.01$) indicated that the gene expression level in the RT group was significant greater or lower than in the IT group; # ($p < 0.05$), ## ($p < 0.01$) indicated that the gene expression level in the RRT group was significant greater or lower than in the RT group.

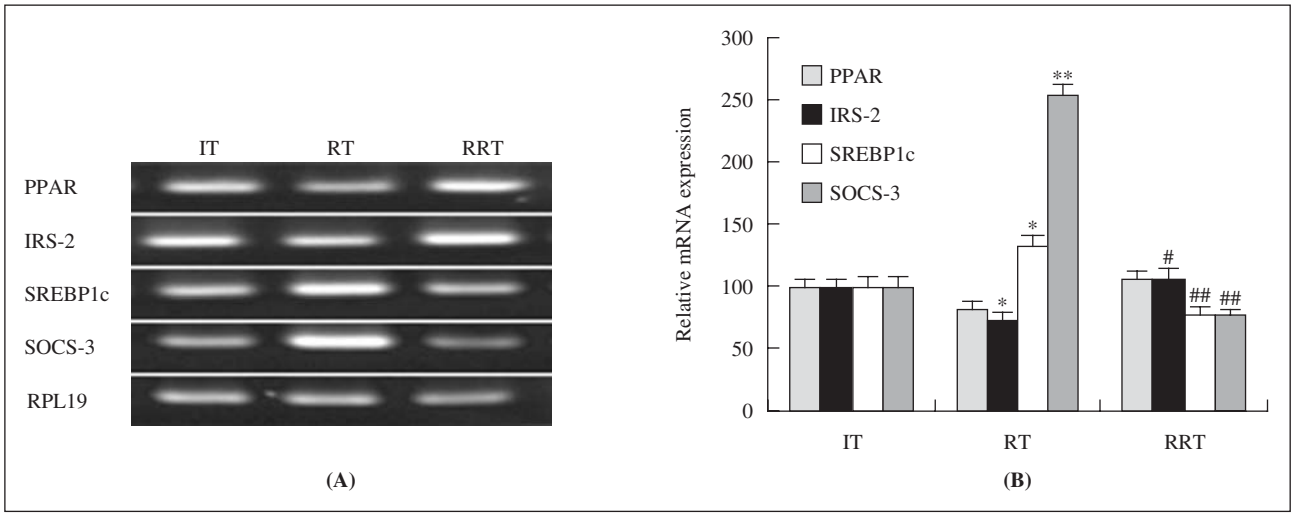


**Figure 4**

Effects of resistin on the insulin signaling components.
After plating, hepatocytes were cultured for 36 h as described in materials and methods. After 36 h, total RNA was extracted and analyzed for the expression of PPARγ, IRS-2, SREBP1c and SOCS-3 (**A**). The quantification of blots for expression of these genes, obtained in 3 independent experiments in triplicate, was shown (**B**). RPL19 was used as an internal control to normalize the expression levels of these genes. * ($p < 0.05$), ** ($p < 0.01$) indicated that the gene expression level in the RT group was significant greater or lower than in the IT group. # ($p < 0.05$), ## ($p < 0.01$) indicated that the gene expression level in the RRT group was significant greater or lower than in the RT group.

repressed expression of IRS-2 (27% reduction, $p < 0.05$) and PPARγ (19% reduction) *(figure 4B)*.

### *Rosiglitazone antagonized the effect of resistin on the glucose metabolic components and the insulin-signaling components*

There is a great deal of evidence that rosiglitazone represses resistin expression at the transcriptional level. However, few studies have investigated the effect of rosiglitazone on resistin action at the post-transcriptional level. In our study, we assayed the relative gene expression in the RRT group. The data showed, for the glucose metabolic components, that rosiglitazone suppressed G6Pase expression (46.9% reduction, $p < 0.05$), whereas it stimulated GLUT2 expression (35.7% induction) compared to the RT group *(figure 3)*; for the insulin-signaling components, rosiglitazone increased expression of PPARγ (30% induction) and IRS-2 (44.5% induction, $p < 0.05$), whereas it decreased expression of SREBP1c (41.5% reduction, $p < 0.01$) and SOCS-3 (69.7% reduction,

$p < 0.01$) compared to the RT group *(figure 4)*. All data proved that rosiglitazone antagonized resistin action at the post-transcriptional level.

## DISCUSSION

Resistin is secreted exclusively by adipocytes in rodents and is thought to play a role in adipocyte differentiation as well as in the development of insulin resistance. In rodent models, mice with impaired resistin function or resistin gene knock-out mice have improved glucose tolerance, insulin sensitivity and active adipogenesis [5, 13], whereas infusion of resistin and resistin overexpression impaired glucose tolerance, insulin action and suppressed adipocyte differentiation [2, 7, 14]. In addition, several transcription factors such as PPARγ, C/EBPα and SREBP, also function in regulating resistin expression at the transcriptional level [15, 16]. The nutritional and hormonal status of animals could also affect resistin expression [17]. So, resistin is presumed to act as a feedback regulator of adipogenesis. Research has shown that the serum resistin protein is increased in obese humans [18]. As we know, resistin is a secretory factor. It should act on target organs *in vivo* in a paracrine or endocrine fashion. In the current study, we mimicked the status by transfecting resistin into L-02 cells and proved that resistin overexpression impairs glucose utilization in hepatocytes. *In vivo* glucose clamp experiments also demonstrated that glucose tolerance was improved significantly in resistin$^{-/-}$ mice, while resistin infusion impaired glucose tolerance in these mice [5].

Rosiglitazone is an anti-diabetic drug which improves insulin sensitivity. In previous studies, rosiglitazone was proved to reverse the effects of resistin by downregulating resistin expression [3, 17]. Interestingly, in this study, this treatment showed the same effect on the status of resistin overexpression, which revealed that rosiglitazone not only regulates resistin expression at the transcriptional level, but also antagonizes resistin action at the post-transcriptional level.

In our study, the data show that overexpression of resistin dramatically represses insulin-stimulated glucose consumption, inhibits expression of PPARγ and IRS-2, but enhances expression of G6Pase, SREBP1c and SOCS-3 in this process. We believed that reduction of PPARγ and IRS-2, and induction of SOCS-3 were bound to impair insulin action, and might cause insulin resistance. Our results coincided with those of Steppan *et al*. They found that resistin could activated SOCS-3 in adipocytes [19]. However in contrast, we showed that resistin had the some action on hepatocytes.

To our knowledge, this is the first report that resistin increases SREBP1c expression. Previously, studies had indicated that SREBP1c, as a transcription factor, could bind resistin promoter and up-regulate resistin expression at the transcriptional level [20]. Our data show that resistin also regulates SREBP1c expression. Considering that SREBP1c directly suppresses IRS-2 transcription [21], the increase in SREBP1c in our study would further block the insulin-signaling pathway. The data suggest that resistin might reduce insulin-stimulated glucose utilization by attenuating the action of insulin. Here, resistin stimulated G6Pase expression, which was accordant with previous research [7]. As the last enzyme in hepatic glucogenesis, an increase in G6Pase would augment glucose output. Considering that resistin did not alter GK activity and GLUT2 expression in our study, it was possible that resistin impaired glucose consumption by enhancing glucose output.

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