1   **Title:** Resistin impairs glucose permeability in EA.hy926 cells by down-regulating GLUT1
2   expression
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Abstract

Type 2 diabetes mellitus (T2DM) is a chronic disease which is now affecting the health of more and more people in the world. Resistin, discovered in 2001, is considered to be closely related to metabolic dysfunction and obesity. Previous study showed that hyperglycemia is always accompanied by a high serum resistin concentration. We therefore investigated whether resistin can mediate glucose transfer across the blood–tissue barrier. Here, we employed a transwell system to analyze glucose permeability in EA.hy926 human endothelial cells treated without or with human resistin. In EA.hy926 cells treated with resistin, the permeability to glucose was heavily impaired. This was due to the down-regulation of GLUT1 expression as a result of the treatment, rather than regulation of tight junctions. In addition, overexpression of *GLUT1* in EA.hy926 cells was able to recover the blocking effect of resistin on glucose permeability. We further found that resistin could inhibit the expression of peroxisome proliferator-activated receptor *gamma* (PPAR γ) and consequently impede the transcription of *GLUT1*. The results of the present study suggested that resistin could cause glucose retention in serum and thus result in hyperglycemia. This provides a novel explanation for hyperglycemia and a potential new way of treating type 2 diabetes mellitus.

Highlight

1. Resistin impaired glucose permeability in EA.hy926 cells.
2. Resistin regulated glucose transportation via affecting transmembrane transport in EA.hy926 cells.
3. Resistin impaired glucose transportation by down-regulating the expression of GLUT1.

4. Resistin down-regulated the expression of GLUT1 by inhibiting PPAR γ .

Abbreviations

T2DM, Type 2 diabetes mellitus; HFD, high fat diet; TJP1, tight junction protein 1; GLUT1, glucose transporter protein 1; PPAR γ , proliferator-activated receptor *gamma*.

Keywords

Resistin, Glucose transportation, GLUT1, PPAR γ

Introduction

As is now well known, obesity is major risk factor for various diseases such as nonalcoholic fatty liver disease, cardiovascular disease, some cancers (McWilliams and Petersen et al., 2009), type-2 diabetes mellitus (Jakobsen, Berentzen et al., 2007), and hypertension (Aneja, El-Atat et al., 2004), among others. Although many studies have revealed related genes and signaling pathways, the underlying mechanism of obesity remains unclear (Larson-Meyer, Heilbronn et al., 2006). Resistin, an adipocytokine which was first identified in 2001 (Steppan, Bailey et al., 2001), has been reported to be involved in many metabolism-associated diseases such as insulin resistance (IR), hyperglycemia, and atherosclerosis (AS) (Kim, Lee et al., 2001). When resistin concentration was lowered by a specific antisense oligodeoxynucleotide in high fat diet (HFD) fed mice, insulin action was considerably improved *in vivo* (Muse, Obici et al., 2004). Accumulated evidence showed that hyperglycemia always accompanied high serum concentrations of resistin (Pagano, Soardo et al., 2006). Endothelial cell layers act as a blood–tissue barrier, regulating transfer of small molecules across the barrier and thus controlling their assimilation by different tissues (Yang, Liu et al., 2014). This raised the question of whether the retention of glucose in plasma was caused by the dysfunction of the endothelial cell layer.

In general, mammalian blood–tissue barriers consisting of endothelial cells regulate the exchange of nutrients between plasma and tissues (Marzesco, Dunia et al., 2002). The paracellular pathway and transmembrane transport are two methods of material transport. The paracellular pathway strictly limits small molecules and ions to diffuse across the paracellular space (Balda and Matter et al., 2008). It relies on the function of tight junctions, which are made up of several proteins including claudins, occludins and tight junction protein 1 (TJP1, also known as ZO-1). All

of these proteins are enriched in endothelial cells (Rubin et al., 1992).

Besides the paracellular pathway, small molecules can transfer across cell layers by transmembrane transport. This relies on a variety of different transmembrane transporters and glucose transporter proteins (GLUTs) facilitate the transfer of glucose across cell membranes. Early studies showed that resistin could inhibit glucose uptake in rat muscle cells (Fan, Gu et al., 2007). This was due to downregulated expression as well as tyrosine phosphorylation of IRS-1 and decreased GLUT4 translocation by resistin (Palanivel, Maida et al., 2006). S-resistin, a non-secretable resistin variant had the similar effect with resistin. Both of them could restrain 3T3-L1 pre-adipocyte differentiation and impair glucose uptake in 3T3-L1 cells by decreasing the expression of GLUT4 (Fernandez, del Arco et al., 2010). GLUT1 (Zhao, Michiue et al., 2015), a core glucose transporter protein which is enriched in the blood–brain barrier, blood–ocular barrier and placental barrier (Tserentsoodol, Shin et al., 1998), facilitates the transendothelial transport of glucose to tissue (Klepper et al., 2015). Impaired S226 phosphorylation in GLUT1 is a characteristic of some GLUT1 deficiency syndromes and causes reduced glucose transport in endothelial cells (Lee, Ma et al., 2015). However, the relationship between resistin and GLUT1 was not very clear.

Since previous studies have indicated that hyperglycemia is accompanied by high serum concentrations of resistin (Li, Yang et al., 2006), we felt it was necessary to further investigate the association between them. Here, we analyzed the effect of resistin on glucose transportation in EA.hy926 cells and investigated the underlying mechanism through which resistin exerted its effect.

Methods

Materials

The total RNA isolation kit was purchased from Omega Bio-Tek (Norcross, GA, USA); the 12-well transwell system was purchased from Corning CoStar (Corning, NY, USA). The mammalian tissue and cell protein extraction kit was bought from Solarbio (Beijing, China). Antibodies against GLUT1, β -Actin, β -Tubulin and OCLN were obtained from Abcam (Cambridge, MA, USA), and recombinant human resistin was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). The glucose assay kit was purchased from Pulilai Biological Technology Co. Ltd. (Beijing, China). Human insulin, rosiglitazone and CY5285 were obtained from Sigma (St Louis, MO, USA). The quantitative protein assay kit was purchased from Thermo-Fisher Scientific (Waltham, MA, USA). The dual luciferase reporter assay kit was bought from Promega (Madison, Wisconsin, USA). The chromatin immunoprecipitation kit was purchased from Millipore (Billerica, MA, USA), while 4 kDa FITC-dextran and 70 kDa FITC-dextran were obtained from Sigma. EA.hy926 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL)

Animal experiments

C57BL/6J male mice (9 weeks old) were purchased from the Model Animal Research Center of Nanjing University. Mice were allowed free access to water and a chow diet and then received a daily vena caudalis injection of mouse resistin (400 ng/mL) for 6 days. The mice were sacrificed after 12h of food withdrawal. All procedures were performed in strict accordance with the

recommendations in the Guide for the Care and Use of Laboratory Animals of Hubei Province. All animal experiments were performed under approval of the Institutional Animal Care and Use Committee (IACUC) of Wuhan University.

Monolayer permeability assay

EA.hy926 cells were seeded into a transwell plate and incubated in a 37°C incubator in an atmosphere of 5% CO₂ for 48 h to ensure that the cells reached complete confluence. At the end of the incubation period the medium was removed from both the upper and lower compartments. Cells on the transwell plate were rinsed with PBS and then 500 µL serum-free medium **with or without 50 ng/mL resistin** was added to the upper compartment, while 1.5 mL serum-free low-glucose medium was added to the lower compartment to maintain the osmotic balance between the upper and lower compartments. After another 24 h, medium was again removed from both the upper and lower compartments and the cells were rinsed with PBS. Next, 500 µL **serum-free high glucose medium** containing 50 ng/mL resistin and 100 nM insulin was added to the upper compartment while 1.5 mL serum-free low glucose medium was added to the lower compartment. The plate was returned to the incubator then 50 µL of the medium in the lower compartment was aspirated at 10 min, 30 min, 1 h, 1.5 h and 2 h for determination of the glucose concentration.

Glucose uptake assay

Glucose uptake is measured as described previously (Osorio-Fuentealba, Contreras-Ferrat et al., 2013). Briefly, EA. hy 926 cells were treated with 100ng/ml resistin for 24h, then cells were washed with Krebs-Ringer buffers three times and incubated with glucose free media containing **200nM** 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino] -2-deoxy-D-glucose (2-NBDG) for 2

hours. Cells were washed again with Krebs-Ringer buffers. The intracellular fluorescence was measured by a fluorescence microplate reader (excitation: 392 nm, emission: 520 nm) and normalized to protein concentration.

Cell proliferation assay

EA.hy926 cells were seeded at a density of 10^4 cells/mL into 12-well plates and incubated in a 37°C incubator in an atmosphere of 5% CO₂ for 24 h. The spent medium was then aspirated and cells were rinsed with PBS, then cells were stimulated without or with 50 ng/mL resistin in fresh medium. Pictures were taken before treatment with resistin (0 h), and at 24 and 48 h after treatment under a 10× phase contrast microscope. At 48 h cells were harvested and counted using a cell counter.

Cell migration assay

The wound healing assay was performed by a classic method that estimates the proliferation as well as migration rates of cells under different culture conditions. In this study, EA.hy926 cells were seeded into 6-well plates and incubated in a 37°C incubator in an atmosphere of 5% CO₂ until cells reached complete confluence. A line was drawn on the underside of the plate as a marker, and then a 200 µL sterile pipet tip was used to scratch three separate wounds perpendicular to the marker line. The cells were then rinsed with PBS and treated without or with 50 ng/mL resistin in fresh medium. Pictures were taken under a 10× phase contrast microscope at 0, 6, 12 and 24 h.

Paracellular flux assay

The assay procedure was broadly similar to the monolayer permeability assay. Briefly, either 4 kDa FITC-dextran or 70 kDa FITC-dextran was added to serum free medium without or with 50

ng/mL resistin and 100 nM insulin to reach a final concentration of 2 mg/mL. At time-points of 10 min, 30 min, 1 h, 1.5 h and 2 h, 50 μ L of the medium in the lower compartment was aspirated and the concentration of FITC-dextran was determined using a fluorescence micro-plate reader (excitation: 392 nm, emission: 520 nm).

Dual luciferase assay

Two deletions of the 5'-flanking region of human *GLUT1* were co-transfected together with PPAR γ into EA.hy926 cells. Twenty-four hours later, cells were lysed and the promoter activity was measured using the dual-luciferase reporter assay system. An illuminometer (GloMax 96 microplate, Promega) was used to assess the enzymatic activity. The *renilla* luciferase reporter plasmid was co-transfected with the two deletions to act as an internal control.

Chromatin immunoprecipitation (ChIP) assays

The assay was carried out in accordance with the instructions of the chromatin immunoprecipitation kit. Briefly, EA.hy926 cells were fixed in 1% formaldehyde and then lysed. Cell lysates were sonicated to obtain chromatin fragments of an average size of 250–500 bp. Samples were dissolved in ChIP dilution buffer and then incubated with PPAR γ antibody overnight. Next day the complexes were precipitated with protein A resin (Millipore) and washed three times. The DNA–protein complexes were eluted from the beads with TE buffer and incubated with proteinase K (Roche, Basel, Switzerland) for 30 mins, then used as the template for quantitative PCR.

Statistical Analysis

All values are presented as means $\pm$ standard deviation. Student's two-tailed *t*-test was used to compare two groups while one-way analysis of variance was used to analyze multiple groups. *P* <

0.05 indicated that differences were statistically significant, while $P < 0.01$ indicated that the differences were highly significant.

Results

Resistin impaired glucose transportation

C57BL/6J mice were treated without or with 400 ng/mL resistin every day for 6 days via tail vein injection, then the body weight as well as the concentration of plasma glucose, insulin and resistin were determined at day 7 (Table. 1). The data showed that resistin administration resulted in higher glucose and insulin levels in plasma. In order to further explore the underlying mechanism, a transwell system was utilized to analyze the permeability of glucose in EA.hy926 cells. The time course study indicated that after incubation with 50 ng/mL resistin and 100 nM insulin, resistin impaired glucose transportation in EA.hy926 cells, an effect which reached significance after 1.5 h (Fig. 1A). And resistin also inhibited glucose uptake in EA.hy926 cells (Fig. 1B-C).

Resistin accelerated the migration and proliferation of EA.hy926 cells, but did not regulate the paracellular pathway.

In order to study how resistin could inhibit glucose permeability in EA.hy926 cells, the monolayer permeability assay was used to investigate paracellular transport because ions and small molecules can diffuse across a monolayer by this route. Since cell proliferation as well as cell migration could cause changes in tight junctions, and increasing the number of cells would compress the spaces between endothelial cells, it was necessary to first investigate the effect of resistin on cell proliferation and cell migration. First, EA.hy926 cells were seeded at

a density of 10^4 cells/mL, treated without or with 50 ng/mL resistin for 24 h, and then used for the cell proliferation assay (Fig. 2A-B). The data showed that resistin could promote the proliferation of EA.hy926 cells. Next, the wound healing assay was performed (Fig. 2C). The result showed that resistin facilitated the migration of EA.hy926 cells. Because cells would stretch out filopodia during their migration, EA.hy926 cells were also stained with phalloidin after treatment with 50 ng/mL resistin for 24 h and the number of filopodia was determined (Fig. 2D-E). Our data showed that resistin stimulated cells to stretch out filopodia, further confirming that resistin facilitated the migration of EA.hy926 cells. The effect of resistin on tight junctions was also investigated. Unexpectedly, the study of the expression levels of *TJPI*, *CLDN* and *OCN* indicated that resistin did not influence their mRNA levels (Fig. 2F) or the protein level of OCLN (Fig. 2G). Moreover, the paracellular flux assay also confirmed that resistin could not affect the diffusion of 4 kDa FITC-dextran or 70 kDa FITC-dextran, two nonionic tracers used to analyze the paracellular pathway (Fig. 2H). Taken together, these results indicated that resistin could not alter tight junctions or the paracellular pathway in EA.hy926 cells. This suggested that resistin mediated glucose transport in other ways.

Resistin impaired glucose transportation via regulation of transmembrane transport in endothelial cells

Transmembrane transportation is another way for ions and small molecules to transfer across a cell monolayer. Glucose transporter protein 1 (GLUT1) plays a pivotal role in transferring glucose across endothelial layers. Consequently we investigated whether resistin could regulate the expression of GLUT1 in EA.hy926 cells. Cells were cultured without or with 50 ng/mL resistin for 24 h, then the expression level of GLUT1 was determined by

qPCR and western blotting. The results showed that resistin inhibited GLUT1 expression (Fig. 3A-C). To further confirm this finding, a *GLUT1* overexpression vector was constructed and transiently transfected into EA.hy926 cells (Fig. 3D-F). Twenty-four hours after the transfection, the glucose permeability assay was performed (Fig. 3G). The data indicated that overexpressing *GLUT1* could block the action of resistin and restore glucose transportation in EA.hy926 cells. Thus, we concluded that resistin inhibited glucose transmembrane transport by down-regulating the expression of GLUT1.

Resistin down-regulated the expression of *GLUT1* by inhibiting PPAR γ

In order to better understand the underlying mechanism by which resistin down-regulated the expression of GLUT1, the effect of resistin on *GLUT1* transcriptional activity was investigated. A 1644 bp 5'-flanking region of human *GLUT1* promoter was acquired via PCR and inserted into the pGL3-basic vector. This plasmid was then transiently transfected into EA.hy926 cells. The dual luciferase assay was utilized to analyze the transcriptional activity of this 5'-flanking region (Fig. 4A). The results revealed that resistin inhibited *GLUT1* transcription (Fig. 4A). *In silico* analysis suggested that STAT3, PPAR γ and MEF2c are the three transcription factors harbored in this region (Fig. 4B). Among these three factors, only PPAR γ was down-regulated by resistin (Fig. 4C), suggesting that resistin down-regulated *GLUT1* expression via suppression of PPAR γ . There are two putative PPAR γ binding sites located at -1484 to -1462 and -1221 to -1199. In order to ascertain which one was the PPAR γ binding site, two mutants containing one or two PPAR γ binding sites were constructed (Fig. 4D). The PPAR γ over-expression vector was co-transfected with these two mutants into EA.hy926 cells, and activity was determined using the luciferase assay (Fig. 4E). The results suggested that PPAR γ could bind to both of the two

putative regions. The chromatin immunoprecipitation (ChIP) assay further confirmed this conclusion (Fig. 4F).

To assess whether resistin down-regulated the expression of *GLUT1* by inhibiting the expression of PPAR γ , a vector overexpressing PPAR γ was transfected into EA.hy926 cells. Six hours after the transfection, EA.hy926 cells were treated without or with 50 ng/mL resistin, and after a further 24 h, the expression of *GLUT1* was analyzed by qPCR (Fig. 4G). The result indicated that PPAR γ could counteract the suppression effect of resistin on *GLUT1* expression. EA.hy926 cells were treated with the PPAR γ agonist rosiglitazone (10 μ M), or the PPAR γ antagonist CY5285 (5 μ M) for 24 h (Fig. 4H). The results showed that rosiglitazone stimulated the expression of *GLUT1* while CY5285 inhibited it, which further verified that PPAR γ could regulate the transcription of *GLUT1*. Overall, resistin suppressed PPAR γ expression and then down-regulated *GLUT1* transcription, causing impaired glucose transportation in EA.hy926 cells.

Discussion

Mounting evidence indicates that high plasma glucose concentrations caused by resistin (Gomez-Diaz, Talavera et al., 2012) are due to the impaired tissue insulin sensitivity which results in diminished glucose absorption (Li, Yang et al., 2006). In fact the dysfunction of endothelial barriers might also result in hyperglycemia, since glucose transport would be inhibited, causing the retention of glucose in plasma. However, few studies have focused on the effect of resistin on glucose transport from blood to tissue. In this study, we show that the permeability of glucose is inhibited after resistin treatment (Fig. 1B). The measurement of intracellular glucose concentration

further confirmed this finding (Fig. 1C). The glucose accumulation in serum caused by the impaired glucose transport in endothelial cells might offer a new explanation for hyperglycemia.

Tight junctions regulate the transfer of ions as well as small molecules across endothelial barriers (Li, Shyu et al., 2015). We also investigated whether resistin affects tight junctions, which play an important role in the conformation of polarized endothelial barriers (Matsuzaki, Takahashi et al., 2010). A previous study indicated that resistin could induce the proliferation and migration of endothelial cells (Calabro, Samudio et al., 2004). In our study, resistin had the same effect on EA.hy926 cells (Fig. 2A-E). This made us wonder whether resistin could also affect tight junctions because increasing numbers of cells would compress the spaces between the endothelial cells. Unexpectedly, the expression of the TJ-related proteins TJP1, OCLN and CLDN were unchanged by resistin treatment (Fig. 2F) and the paracellular pathway was also not affected (Fig. 2E). Our data show that resistin does not regulate tight junctions or the paracellular pathway, suggesting that resistin might mediate glucose transport in other ways.

Next, the effect of resistin on transmembrane transport was analyzed. Our data show that resistin down-regulates expression of GLUT1 (Fig. 3A-B) and that overexpressing *GLUT1* in EA.hy926 cells completely blocks the role of resistin (Fig. 3C-D). Based on these results, we hypothesized that resistin impairs glucose transport by suppressing the expression of *GLUT1*. A similar effect has been reported by Pifferi et al., who found that impaired glucose transport in the brain of rats was due to decreased GLUT1 expression in the endothelial cells of the blood–brain barrier (Pifferi, Jouin et al., 2007).

Peroxisome proliferator-activated receptor *gamma* (PPAR γ), encoded by the *PPARG* gene in humans, is a type II nuclear receptor that is primarily present in macrophages (Desvergne and

Wahli et al., 1999), colon and adipocytes (Devine, Eubank et al., 1999). A great deal of research has shown that PPAR γ can regulate glucose metabolism (Mudaliar and Henry et al., 2001) and adipocyte differentiation (Rosen, Sarraf et al., 1999). It is also closely tied with cancer as well as arteriosclerosis (Marx, Kehrle et al., 2002). Rosiglitazone, widely used in the treatment of diabetes (Phielix, Szendroedi et al., 2011), is a classical type of hypoglycemic drug which exerts its effect by activating PPAR γ to lower plasma glucose (Hulstrom, Hojlund et al., 2008). It works by combining with the PPARs and activating the expression of related genes to promote glucose uptake in tissues, thus reducing plasma glucose. In our study, *in silico* analysis suggested that there were two putative PPAR γ binding regions in *GLUT1* gene (Fig. 4B). Since resistin is an adipocytokine, this prompted us to investigate whether resistin could regulate the expression of GLUT1 via transcriptional regulation. Our results suggest that resistin suppresses the expression of PPAR γ (Fig. 4C). Both the dual luciferase assay and the ChIP assay confirmed that PPAR γ can bind the two putative regions (Fig. 4D-F). Overexpressing PPAR γ in EA.hy926 cells recovered the suppression effect of resistin on *GLUT1* expression (Fig. 4G). This indicates that resistin down-regulates GLUT1 expression through the suppression of PPAR γ . Rosiglitazone facilitates glucose uptake in adipose tissue, liver and muscle. Our research shows that rosiglitazone promotes the expression of *GLUT1* (Fig. 4H). Given that PPAR γ expresses in vascular endothelial cells, this suggests that rosiglitazone could also induce endothelial cells to transport plasma glucose and could thus constitute a novel anti-diabetes therapy.

In conclusion, we show that resistin down-regulates the expression of *GLUT1* by suppressing PPAR γ , thus causing impaired glucose transportation in endothelial cell layers. Although our research was based on resistin, hyperglycemia induced by other factors might share a similar

mechanism. This means that the disorder of endothelial cells could prevent glucose transferring across the blood–tissue barrier, and provides a novel explanation for hyperglycemia and a new therapeutic option for type 2 diabetes mellitus.

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Author Contributions

QL, XC, JH, XY, JS performed experiments. QL analyzed the data. ZY and LZ conceived and supervised the study. QL and LZ wrote the paper. All authors reviewed the manuscript and approved the final version of the manuscript.

Competing financial interests

None

Table.1 Body weight, the concentration of plasma glucose, resistin and insulin.

	Control Group (n=5)	Resistin Administered Group (n=5)
Glucose, mM	5.95±0.25	7.65±0.75 **
Insulin, mU/L	144.78±11.3	184.76±15.15 **
Resistin, ng/ml	6.63±0.2	9.14±0.6 **
Weight, g	22.37±1.19	22.64±1.08

Mice were treated with resistin for 6 days, as described in Materials and Methods. ** $P < 0.01$.

Figure legends

Fig. 1 Resistin impaired glucose permeability.

A. EA.hy926 cells were treated without or with 50 ng/mL human resistin together with 100 nM insulin and the glucose concentration in the lower compartment were determined. B. At 24 h after treatment, glucose uptake assay was performed. C. Nuclei appear in blue and 2-NBDG singals in green. Data are represented as mean $\pm$ SD, * $P < 0.05$; ** $P < 0.01$.

Fig. 2. Resistin did not affect tight junctions

A. EA.hy926 cells were seeded at a density of 10^4 cells/mL. Twenty-four hours later, cells were treated without or with 50 ng/mL resistin, and pictures were taken before the addition of resistin (0 h), and at 24 and 48 h after treatment under a 10 $\times$ phase contrast microscope. B. Cell numbers were counted at 48 h using a cell counter. C. EA.hy926 cells were seeded into a 6-well plate and cultured until they reached complete confluence. Then three separate wounds were scratched using a sterile 200 μ L pipet tip. Cultures were then treated without or with 50 ng/mL resistin for 0, 6, 12,

and 24 h and a series of pictures were taken at each time point. D. Cells were stained with phalloidin. E. the numbers of filopodia were quantified. F. Effect of resistin on gene transcription of TJ-related genes. G. The protein level of OCLN. H: Paracellular flux of 4 kDa FITC-dextran (FD4) and 70 kDa FITC-dextran (FD70) in the lower compartment was determined using a fluorescence micro-plate (excitation: 392 nm, emission: 520 nm). Data are represented as mean $\pm$ SD, * P < 0.05; ** P < 0.01.

Fig. 3. Resistin down-regulated the expression of GLUT1.

A. The relative mRNA level of *GLUT1* in EA.hy926 cells was reduced after treatment with 50 ng/mL resistin. B. The relative protein level of GLUT1 in EA.hy926 cells was also reduced after treatment with 50 ng/mL resistin. C. The quantitative analysis of figure B. D. Transfection efficiency of *GLUT1* in EA.hy926 cells. E. The relative protein level of GLUT1 in EA.hy926 cells overexpressing *GLUT1* gene. F. The quantitative analysis of figure E. G. *GLUT1* overexpression restored the blocking effect of resistin on glucose permeability. Data are represented as mean $\pm$ SD, * P < 0.05; ** P < 0.01.

Fig. 4. Resistin reduced the expression of PPAR γ .

A. The 1644 bp 5'-flanking region of human *GLUT1* contains the promoter activity, which could be inhibited by resistin. B. *In silico* analysis of putative transcription factor binding sites. C. The effect of resistin on the mRNA levels of different putative transcription factors. D. Analysis of PPAR γ binding regions. E. The binding regions of PPAR γ were confirmed by the dual luciferase

480 assay. The internal control was a CMV-driven *Renilla* luciferase reporter plasmid and the
481 pcDNA-3.1 vector used as a negative control. F. The binding region of PPAR γ was confirmed by
482 ChIP assay in EA.hy926 cells. G. Overexpression of PPAR γ in EA.hy926 cells restored the
483 suppressing effect of resistin on *GLUT1* expression. H. The agonist of PPAR γ , rosiglitazone (10
484 μ M) stimulated the expression of *GLUT1* while the PPAR γ antagonist CY5285 (5 μ M) inhibited
485 its expression. Data are represented as mean $\pm$ SD, * P < 0.05; ** P < 0.01.

Fig. 1

Fig. 2

Fig. 3

A

B

C

D

F

G

pcDNA3.1	+	+	-	-
pcDNA3.1- <i>glut1</i>	-	-	+	+
Resistin	-	+	-	+

E

Fig. 4