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Resistin impairs glucose permeability in EA.hy926 cells by down-regulating GLUT1 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* (PPARγ) 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.

1. 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, 2009), type-2 diabetes mellitus (Jakobsen et al., 2007), and hypertension (Aneja et al., 2004), among others. Although many studies have revealed related genes and signaling pathways, the underlying mechanism of obesity remains unclear (Larson-Meyer et al., 2006). Resistin, an adipocytokine which was first identified in 2001 (Steppan et al., 2001), has been reported to be involved in many metabolism-associated diseases such as insulin resistance (IR), hyperglycemia, and atherosclerosis (AS) (Kim 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 et al., 2004). Accumulated evidence showed that hyperglycemia always accompanied high serum concentrations of resistin (Pagano 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 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 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, 2008). It relies on the function of tight junctions,

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

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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, 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 et al., 2007). This was due to downregulated expression as well as tyrosine phosphorylation of IRS-1 and decreased GLUT4 translocation by resistin (Palanivel et al., 2006). S-resistin, a non-secretable resistin variant had the similar effect with resistin. Both of them could restrain 3T3-L1 preadipocyte differentiation and impair glucose uptake in 3T3-L1 cells by decreasing the expression of GLUT4 (Fernandez et al., 2010). GLUT1 (Zhao et al., 2015), a core glucose transporter protein which is enriched in the blood–brain barrier, blood–ocular barrier and placental barrier (Tserentsoodol et al., 1998), facilitates the transendothelial transport of glucose to tissue (Klepper, 2015). Impaired S226 phosphorylation in GLUT1 is a characteristic of some GLUT1 deficiency syndromes and causes reduced glucose transport in endothelial cells (Lee 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 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.

2. Methods

2.1. 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).

2.2. 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.

2.3. Monolayer permeability assay

EA.hy926 cells were seeded into a transwell plate and incubated in a 37 °C incubator in an atmosphere of 5% CO_2 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.

2.4. Glucose uptake assay

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

2.5. 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% CO_2 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.

2.6. 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% CO_2 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.

2.7. 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