Resistin Exacerbates Insulin Resistance under the Condition of Low Adiponectin in 3T3-L1 Adipocytes

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Abstract

Adipocytokines such as resistin, TNF-α, and adiponectin, which are adipocyte-derived peptides, play important roles in glucose metabolism. Resistin and TNF-α have been implicated as factors associated with the development of insulin resistance in obesity. In contrast, adiponectin has been shown to improve insulin sensitivity in insulin resistance. However, the interaction among these adipocytokines is still unknown. In this study, we investigated the mechanism of the effects of these 3 adipocytokines (resistin, adiponectin, and TNF-α) on glucose transport in 3T3-L1 adipocytes.

Glucose uptake was evaluated by 2-[3H] deoxy-glucose (DOG) uptake assay in 3T3-L1 adipocytes. Resistin and adiponectin secretion were analyzed by western blotting.

Adenovirus-mediated overexpression of resistin inhibited insulin-stimulated 2-DOG uptake by only 15% compared with control cells. In contrast, pretreatment of cells with 10 ng/mL TNF-α for 3 hrs did not inhibit insulin stimulated 2-DOG uptake compared with control cells, whereas overexpression of resistin led to a ~ 40% decrease in insulin stimulated 2-DOG uptake following pretreatment with TNF-α. TNF-α has been shown to suppress the expression and secretion of adiponectin from adipocytes. Therefore, we speculated that this potentiating effect of resistin might be caused by the reduction in adiponectin secretion. We confirmed that the secretion of adiponectin was decreased by ~ 50% in TNF-α treated cells compared to control cells. Furthermore, overexpression of adiponectin prevented this additive effect of resistin and TNF-α.

In conclusion, these results suggest that: (1) TNF-α enhances the action of resistin via the reduction of adiponectin, 2) Resistin may cause severe insulin resistance under low adiponectin levels.

Keywords: Resistin; Adiponectin; TNF-α

Abbreviations: TNF-α: Tumor Necrosis Factor-α; AMPK: Activated Protein Kinase; Ad-resistin: Resistin Adenovirus; Ad-adipo: Adiponectin Adenovirus; Ad-lacZ: lacZ Adenovirus; 2-DOG: 2-deoxyglucose; IR: Insulin Receptor; IRS-1: Insulin Receptor Substrate 1

Introduction

Obesity is linked to common metabolic diseases including insulin resistance, which constitutes a principal risk factor for type 2 diabetes. Accumulating evidence indicates that changes in adipose-secreted factors in obesity, including release of inflammatory cytokines, dramatically affect insulin sensitivity [1-3].

Resistin is a 12.5 kDa cysteine-rich polypeptide discovered in a screen for adipocyte gene products that are downregulated by antidiabetic thiazolidinedione drugs [4]. Resistin serum levels increase in diet-induced and genetic models of obesity [4], although adipose tissue mRNA levels are reduced [5,6]. Systemic treatment or transgenic overexpression of resistin in rodents decreases the ability of insulin to suppress hepatic glucose production [7,8]. Conversely, ablation of the resistin gene or reduction in resistin protein through antisense oligonucleotide treatment improves insulin sensitivity through AMP-activated protein kinase (AMPK) activation [9,10]. The resistin receptor is not known but the effect of resistin inducing insulin resistance is associated with attenuation of AMPK phosphorylation [8-10]. Human resistin is made and secreted by macrophages [11]. Plasma resistin levels and single-nucleotide polymorphisms has been linked to obesity and lipid and glucose abnormalities in some studies [12-15], although others have failed to establish such a relationship [16].

On the other hand, adiponectin [17-20] is a hormone secreted by adipocytes, which functions as the key antidiabetic and anti-atherogenic adipocytokine [21]. Plasma adiponectin levels are decreased in obesity, insulin resistance, and type 2 diabetes mellitus [22]. Many studies have shown that high levels of adiponectin are associated with insulin sensitization, whereas low levels are found in insulin resistance [23-27]. This insulin-sensitizing effect of adiponectin is mediated by the inhibition of gluconeogenesis [27] and the stimulation of fatty acid oxidation [25,26] via activation of AMPK [28]. In contrast, TNF-α, an adipocyte-tissue derived peptide, is markedly elevated in obese or diabetic animals and humans [29]. In a mouse model, adiponectin deficiency was associated with severe diet-induced insulin resistance and elevated TNF-α levels by not resistin levels [30]. Thus, adiponectin and TNF-α are reciprocally expressed in adipocytes and have opposing effects on the regulation of insulin resistance. However, the interaction among these 2 adipokines and resistin is still unknown.

In this study, we investigated the mechanism of the effects of these adipocytokines on glucose transport in 3T3-L1 adipocytes.

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Materials and Methods

Materials

3T3-L1 preadipocytes were purchased from American Type Cell Collection (Manassas, VA, USA). pAxC-Awt plasmid vector was purchased from TAKARA Biomedical (Shiga, Japan). Dulbecco’s modified Eagle’s medium (DMEM, high glucose), streptomycin, trypsin, fetal bovine serum, TRIZol reagent, pCR2.1-TOPO vector, lithium dodecyl sulfate sample buffer, and Sample Reducing Agent were purchased from Invitrogen (Carlsbad, CA, USA). The RNeasy kit was purchased from QIAGEN Inc. (Valencia, CA, USA). Mouse TNF-α was purchased from R&D System (Minneapolis, MN, USA). Anti-resistin antibody (ab93069) was purchased from Abcam (Tokyo, Japan). Anti-adiponectin antibody was purchased from Affinity BioReagents, (Golden, CO, USA). Polyvinylidene difluoride (PVDF) transfer membranes were purchased from Millipore Corp. (Bedford, MA, USA). iScript cDNA Synthesis Kit and iQ SYBR Green Supermix were purchased from Bio-Rad Laboratories (Richmond, CA, USA). 2-[^H] deoxyglucose (DOG) was purchased from PerkinElmer Inc. (Waltham, MA, USA). Adiponectin was purchased from Affinity BioReagents (Golden, CO, USA).

Subcloning of the mouse resistin and adiponectin cDNA by RT-PCR

Total RNA was extracted from differentiated 3T3-L1 adipocytes using TRIzol reagent (Invitrogen). Briefly, 1 g of total RNA was reverse-transcribed with 200 U of reverse transcriptase using the Superscript III kit (Invitrogen) according to the manufacturer’s recommendations. The mouse resistin cDNA (359 bp) and full-length adiponectin cDNA (765 bp) were amplified using the 5’-GTACCCACGGGATGAAAGACC-3’ sense and 5’-ACATCGAGAACGCTGCAG-3’ antisense primers, and the 5’-CAGGATGCTACTGTTGCAAGC-3’ sense and 5’-TGGTAGTGGTGACGTCAAGG-3’ antisense primers, respectively. PCR products were separated on a 2% agarose gel and purified using the Qiagen PCR Purification Kit (QIAGEN, Valencia, CA). The mouse resistin cDNA and adiponectin cDNA were thereafter subcloned into the pCR2.1-TOPO (Invitrogen), respectively, and sequenced to confirm that the clones corresponded to mouse resistin (GenBank accession number AF323080) and mouse adiponectin (GenBank accession number AF304466).

Construction of recombinant adenoviruses

Resistin adenovirus (Ad-resistin), adiponectin adenovirus (Ad-adipo), and lacZ adenovirus (Ad-lacZ) were generated and purified using a previously described protocol [8]. Briefly, the mouse resistin cDNA was inserted in the pAxC-Awt plasmid to generate pAxC-Awt-mouse resistin. The resulting plasmid, which contains the resistin cDNA under the control of a CAG promoter (CMV enhancer, chicken β-actin promoter, and parts of an untranslated region of rabbit β-globin), was transfected into HEK 293 cells. Recombinant adenovirus from a single plaque was propagated in HEK 293 cells and purified with Adeno-X Virus Purification and Rapid Titer Kits (TAKARA BIO INC, Shiga, Japan). Ad-adipo and control Ad-lacZ, which carries the β-galactosidase cDNA were isolated using the same procedure, respectively. These recombinant viruses were dialyzed in phosphate-buffered saline, pH 7.4, and stored in 10% glycerol/phosphate-buffered saline at -80°C until use.

Cell culture and cell treatment

3T3-L1 cells were cultured and differentiated as described previously [31]. For adenovirus infection, differentiated 3T3-L1 adipocytes were transduced for 2 hrs in DMEM (high glucose) with 2% heat-inactivated fetal bovine serum with recombinant adenovirus of resistin, or/and, adiponectin, or/and control recombinant adenovirus of lacZ. Transduced cells were incubated for 48 hrs at 37°C in 10% CO2 and DMEM (high glucose) with 10% heat-inactivated serum, followed by incubation in starvation media required for the assays.

2-Deoxyglucose uptake assay

Glucose uptake was initiated as described previously [31], with some modifications. After 48 hrs of adenovirus infection, 3T3-L1 adipocytes were serum-starved for 6 hrs and then stimulated with 100 ng/ml insulin in KRP-HEPES buffer (10 mM HEPES, pH 7.4, 131.2 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 25 mM NaH2PO4) for 30 min at 37°C. Glucose uptake was determined in triplicate at each point after the addition of 2-[^H] DOG (0.1 μCi, final concentration 0.1 mM) in KRP-HEPES buffer for 5 min at 37°C. The cells were washed 3 times in ice-cold phosphate-buffered saline and solubilized in 1 N NaOH. Each sample was subjected to liquid scintillation counting [31].

Immunoblotting analysis

Differentiated 3T3-L1 adipocytes were transduced with Ad-resistin, Ad-adipo, or/and Ad-lacZ. At 48 hrs after infection, 3T3-L1 adipocytes were starved for 3 hrs prior to treatment without or with TNF-α (10 ng/mL) for 3 h. Conditioned medium was then collected. For western blot analysis, condition medium (10 μl per lane) was denatured by boiling in lithium dodecyl sulfate sample buffer and Sample Reducing Agent (Invitrogen), and then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were transferred to a polyvinylidene difluoride membrane (Immobilon-P), using a semi-dry Transblot apparatus (Bio-Rad Laboratories Inc.). For immunoblotting, membranes were blocked and probed with resistin (Abcam) or adiponectin (Affinity BioReagent) antibodies. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies before chemiluminescence detection, according to the manufacturer’s instructions (Pierce, Rockford, IL, USA). Band intensities were quantified by densitometry using the Image J software (NIH, USA).

Statistical analysis

Statistical analysis were performed using Excel statistics 2008 (SSRI, Tokyo, Japan) added in Excel software (Microsoft Corporation, USA). The data were analyzed by one-way ANOVA or unpaired two-tailed Student t test. A P-value of less than 0.05 was considered to indicate significance. All values are expressed as mean ± SE.

Results

Resistin inhibits insulin-stimulated 2-DOG uptake in 3T3-L1 adipocytes

We first examined the effects of resistin overexpression on insulin-stimulated glucose uptake in 3T3-L1 adipocytes. As shown in figure 1, overexpression of resistin by Ad-resistin led to a 15% decrease (P<0.05) in insulin (100 ng/mL)-stimulated 2-DOG uptake, whereas resistin did not induce any change in basal 2-DOG uptake.

TNF-α exacerbates the effect of resistin on insulin-stimulated 2-DOG uptake in 3T3-L1 adipocytes

Pretreatment of cells with 10 ng/mL TNF-α for 3 hrs did not inhibit insulin stimulated 2-DOG uptake compared with control cells, whereas
overexpression of resistin led to an approximately 40 % decrease (P<0.01) in insulin (100 ng/mL)-stimulated 2-DG uptake following pretreatment with TNF-α (Figure 2).

**TNF-α suppresses the secretion of adiponectin from 3T3-L1 adipocytes**

TNF-α has been reported to suppress the expression and secretion of adiponectin from adipocytes. It has been known that adiponectin is an insulin-sensitizing hormone, which is also secreted from adipocytes. We, therefore, speculated that the glucose uptake-suppressing effect of resistin that is induced by TNF-α may be caused by the reduction of adiponectin. To prove this, we confirmed whether TNF-α suppresses the secretion of adiponectin in 3T3-L1 adipocytes by western blotting analysis. The supernatants after treatment with or without TNF-α for 3 hrs were used as samples. As shown in figure 3, TNF-α suppressed the secretion of adiponectin in 3T3-L1 whether resistin was overexpressed or not.

**Overexpression of adiponectin prevented the synergic effect of resistin and TNF-α in 3T3-L1 adipocytes**

Next, we investigated whether this effect is improved by the overexpression of adiponectin. The overexpression of adiponectin prevented the resistin and TNF-α induced inhibition of insulin-stimulated 2-DG uptake (Figure 4). Thus, the TNF-α-induced effect of resistin is improved by the overexpression of adiponectin.

**Reduced adiponectin secretion by treatment with TNF-α is rescued by infection of 3T3-L1 adipocytes with Ad-adipo**

Furthermore, we examined the adiponectin levels by western blotting analysis. For this, we collected the supernatants of cells overexpressing resistin or both adiponectin and resistin after treatment with or without TNF-α for 3 h. The adiponectin levels, which were decreased by TNF-α treatment, improved by the overexpression of adiponectin (Figure 5).

**Discussion**

The major finding of this study is that low adiponectin levels leads to
Adiponectin and type 2 diabetes [35]. Thus, adiponectin is negatively associated with insulin resistance in both humans and rodents. Circulating adiponectin levels are reduced in obesity [32,33] and, most importantly, visceral fat accumulation [34]. The serum concentration of adiponectin is inversely correlated with body mass index (BMI) [32,33]. Adiponectin is upregulated in obese states model [4], and systemic treatment or transgenic overexpression of resistin in rodents decreases the ability of insulin to suppress hepatic glucose production [7,8]. In some human studies, plasma resistin levels and single-nucleotide polymorphisms have been linked to obesity and lipid and glucose abnormalities [12-15], while in some others was not [16]. Thus, the action of resistin in humans is controversial. The reason for the discrepancy in the effect of resistin in many human studies is not yet understood. As a speculation, it could be mentioned, that altered adiponectin levels could lie behind these discrepancies, based on this study.

On the other hand, adiponectin, which has an opposing effect to resistin, enhances insulin sensitivity in liver and skeletal muscle, via an AMPK-dependent pathway in rodent models [24,28]. In humans, the serum concentration of adiponectin is inversely correlated with body mass index (BMI) [32,33] and, most importantly, visceral fat accumulation [34]. Circulating adiponectin levels are reduced in obesity and type 2 diabetes [35]. Thus, adiponectin is negatively associated with insulin resistance in both humans and rodents. Adiponectin reaches a plasma concentration of 2 to 10 µg/mL in humans [36], whereas plasma resistin levels are around 25 ng/mL in obese patients with diabetes [37]. Thus, adiponectin is the most abundant protein secreted by white adipose tissue. Therefore, we have hypothesized that the effect of resistin depends on the concentration of adiponectin. It has been reported that the expression and secretion of adiponectin is regulated by a variety of hormones and cytokines that influence insulin sensitivity in adipocytes [38,39].

TNF-α, a cytokine that induces insulin resistance, functions as a major negative regulator of adiponectin and causes metabolic disorders by impairing the function of adipose tissues [40]. Due to the protective effect of adiponectin against insulin-associated metabolic diseases, TNF-α-induced reduction of adiponectin expression is considered a crucial event in the development of insulin resistance. It is well documented that 3- to 4 days of exposure of 3T3-L1 adipocytes to TNF-α causes insulin resistance [41] and that a large decrease in the GLUT4 content plays a major role in the decrease in insulin-stimulated glucose transport [42,43]. Moreover, it has been reported that long-term exposure to TNF-α causes insulin resistance and a decrease in insulin receptor and insulin receptor substrate 1 tyrosine phosphorylation in response to a maximal insulin stimulus in Fao hepatoma cells [44] and L6 myocytes [45], which do not secrete adiponectin. These reports suggest that long-term exposure to TNF-α directly causes insulin resistance.

The data showed that resistin exacerbates the insulin-stimulated glucose uptake under the condition of low adiponectin levels in 3T3-L1 adipocytes. Resistin is upregulated in obese states model [4], and systemic treatment or transgenic overexpression of resistin in rodents decreases the ability of insulin to suppress hepatic glucose production [7,8]. In some human studies, plasma resistin levels and single-nucleotide polymorphisms have been linked to obesity and lipid and glucose abnormalities [12-15], while in some others was not [16]. Thus, the action of resistin in humans is controversial. The reason for the discrepancy in the effect of resistin in many human studies is not yet understood. As a speculation, it could be mentioned, that altered adiponectin levels could lie behind these discrepancies, based on this study.

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resistance independent of adiponectin. However, it is still unclear whether short-term treatment with TNF-α causes insulin resistance in 3T3-L1 adipocytes. Therefore, in this study we investigated the short-term effect of TNF-α on insulin-stimulated 2-DOG uptake in 3T3-L1 adipocytes. Our results show that short-term exposure to TNF-α does not affect insulin-stimulated glucose uptake in 3T3-L1 adipocytes, whereas TNF-α increased glucose production. J Clin Invest 114: 225-230.

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