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Transmembrane tumor necrosis factor-alpha sensitizes adipocytes to insulin

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ABSTRACT

Transmembrane TNF-α (tmTNF-α) acts both as a ligand, delivering 'forward signaling' via TNFR, and as a receptor, transducing 'reverse signaling'. The contradiction of available data regarding the effect of tmTNF-α on insulin resistance may be due to imbalance in both signals. Here, we demonstrated that high glucose-induced impairment of insulin-stimulated glucose uptake by 3T3-L1 adipocytes was concomitant with decreased tmTNF-α expression and increased soluble TNF-α (sTNF-α) secretion. However, when TACE was inhibited, preventing the conversion of tmTNF-α to sTNF-α, this insulin resistance was partially reversed, indicating a salutary role of tmTNF-α. Treatment of 3T3-L1 adipocytes with exogenous tmTNF-α promoted insulin-induced phosphorylation of IRS-1 and Akt, facilitated GLUT4 expression and membrane translocation, and increased glucose uptake while addition of sTNF-α resulted in the opposite effect. Furthermore, tmTNF-α downregulated the production of IL-6 and MCP-1 via NF-κB inactivation, as silencing of A20, an inhibitor for NF-κB, by siRNA, abolished this effect of tmTNF-α. However, tmTNF-α upregulated adiponectin expression through the PPAR-γ pathway, as inhibition of PPAR-γ by GW9662 abrogated both tmTNF-α-induced adiponectin transcription and glucose uptake. Our data suggest that tmTNF-α functions as an insulin sensitizer via forward signaling.

1. Introduction

Transmembrane TNF-α (tmTNF-α) is a type II transmembrane protein. Its extracellular segment is cleaved by membrane-bound metalloproteases, chiefly TNF-α-converting enzyme (TACE), releasing soluble TNF-α (sTNF-α). Both forms of TNF-α exert their biological functions via binding to TNF receptors (TNFRs) (Black et al., 1997). Although sTNF-α has been widely recognized as a link between adiposity and insulin resistance, a few studies have shown that tmTNF-α is also bioactive in adipocytes and is involved in obesity-related insulin resistance. Xu et al. demonstrated that tmTNF-α inhibits adipocyte differentiation *in vitro* (Xu et al., 1999), and that its expression is significantly increased in the adipose tissue in different rodent obesity models as well as in obese humans (Xu et al., 2002b). Suppressing ectodomain shedding of tmTNF-α by the TACE inhibitor KB-R7785 shows an antidiabetic effect (Morimoto et al., 1997). Similarly, mice heterozygous for TACE (*Tace*$^{+/-}$) resulting in increased expression of tmTNF-α were relatively protected from obesity and insulin resistance (Serino et al., 2007). These data suggest that tmTNF-α, unlike sTNF-α, may promote insulin sensitivity. However, it has also been reported that adipocyte specific expression of noncleavable tmTNF-α impaired local insulin sensitivity and decreased whole body adipose mass in a transgenic mouse model (Xu et al., 2002a). When this noncleavable tmTNF-α mutant was expressed in multiple different organs, it led to increased weight gain and adipose tissue mass of mice fed a high-fat diet (Voros et al., 2004).

The discrepancy of the reported roles of tmTNF-α in insulin sensitivity may be associated with the balance of bidirectional signaling of tmTNF-α. tmTNF-α as a ligand delivers 'forward signaling' via TNFR to target cell or, as a receptor, transduces 'reverse signaling' to its expressing cell. Since both tmTNF-α and TNFR are substrates of TACE (Scheller et al., 2011), inhibition of TACE not only increases expression of tmTNF-α and TNFR on the cell surface, but also inhibits release of these soluble molecules. tmTNF-α binds TNFR and transduces bidirectional signals simultaneously via tmTNF-α and TNFR respectively. In noncleavable tmTNF-α transgenic mice, TNFR but

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not tmTNF-α can still be cleaved by TACE that is upregulated in obesity (Xu et al., 2002b). These increased soluble TNFRs bind to tmTNF-α to deliver reverse signaling, meanwhile these soluble molecules can competitively decrease interaction of tmTNF-α and cell-surface TNFRs to disrupt forward signaling. If this were the case, the beneficial effect of TACE inhibitor would be attributed to the forward signaling of tmTNF-α. Furthermore, none of those experimental systems was sufficient to distinguish the actions of tmTNF-α via forward signaling from those via reverse signaling.

Obesity is considered to be a chronic low-grade inflammatory state that results in insulin resistance (Emanuela et al., 2012). Because we previously showed that exogenous tmTNF-α inhibits NF-κB activation (a pathway associated with inflammation) in the neutrophil-like cell line HL-60 (Chen et al., 2011), we hypothesized that tmTNF-α may play a role in sensitizing insulin-response via its forward signaling. In the present study, we directly treated 3T3-L1 and human primary adipocytes with exogenous tmTNF-α and found that tmTNF-α inhibited the production of proinflammatory adipokines and promoted the release of anti-inflammatory adipokine, increasing insulin sensitivity of adipocytes via forward signaling.

2. Materials and methods

2.1. Preadipocyte isolation and adipogenic differentiation

Human preadipocytes were isolated from the freshly excised abdominal subcutaneous adipose tissue of seven healthy women (aged 25–42 years, with BMI of 21.8 ± 2.6 kg/m^2) after liposuction or abdominoplasties at the Department of Plastic Surgery, Tongji Hospital. This study was conducted in accordance with the guidelines of the local ethics committee. Adipose tissue was minced and digested by 1 mg/ml type II collagenase (Grand Island, NY) at 37 °C for 60 min under constant shaking. After filtration through a double-layered sterile gauze, the filtrates were centrifuged at 1000 rpm for 10 min. After lysis of erythrocytes, the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% Fungizone.

Once confluence of human preadipocytes was reached, adipogenic differentiation was induced for 3 days in OriCell™ Adipogenic Differentiation Medium A, and then in matched Medium B (Cyagen, Goleta, CA) for another 24 h. This was repeated 3 times. The cells were then maintained in Medium B for an additional 7–9 days until accumulated visible lipid droplets emerged.

3T3-L1 murine fibroblast cell line was grown to confluence in DMEM supplemented with 10% FBS at 37°C. Adipogenic differentiation of 3T3-L1 was induced by exposure to 0.5 mM IBMX (3-isobutyl-1-methylxanthine, Sigma-Aldrich, St. Louis, MO), 1 μM dexamethasone, and 10 μg/ml insulin for 2 days, and then to 10 μg/ml insulin alone for additional 2 days. Thereafter, the cells were maintained in the medium containing 10% FBS until >90% of the cells showing accumulated lipid vacuoles in the cytoplasm by staining with Oil Red O.

2.2. Stimulation of adipocytes with both forms of TNF-α

Fully differentiated 3T3-L1 adipocytes or primary human adipocytes as target cells were treated for 24 h with 20 ng/ml sTNF-α (Peprotech, Rocky Hill, NJ) or tmTNF-α expressed at a high level by Raji cells as effector cells (Zhang et al., 2008), a malignant B-cell line had been fixed with 4% paraformaldehyde for 30 min at room temperature (RT), at an effector/target (E/T) ratio of 10:1. The untreated adipocytes served as a control. For identification of the specific actions of tmTNF-α, 4% paraformaldehyde-fixed Raji cells were treated with an anti-TNF-α antibody (BD Pharmingen, San Jose, CA) for 1 h to neutralize tmTNF-α prior to addition to the target adipocytes. For glucose uptake assay or test of insulin signaling, 100 nM of insulin was added to the cells and incubated for 30 min after TNF-α-stimulation.

2.3. Glucose uptake assay

3T3-L1 adipocytes or primary human adipocytes were treated for 24 h with different concentrations of glucose and 1 μM insulin, or the two forms of TNF-α. After re-equilibration in serum- and glucose-free media for 2 h, 1×10^6 adipocytes were incubated for 30 min with or without 100 nM of insulin in glucose-free Krebs–Ringer Hepes (KRH) buffer. Glucose uptake was detected by the addition of 2-[1,2-^{3}H]-deoxy-D-glucose (0.5 μCi/ml) for 10 min, followed by measurement of the radioactivity of the cell lysates after solubilization in 0.1 M NaOH, by a liquid scintillation counter (Perkin Elmer). Specific uptake was obtained by subtracting nonspecific deoxyglucose uptake that was determined in the presence of 20 μM cytochalasin B, from each of the resultant values (Zhou et al., 2010).

2.4. Western blot analysis

Adipocytes were harvested and lysed in ice-cold lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The total protein was obtained after centrifugation at 12,000 rpm for 20 min at 4 °C. For detection of GLUT4 translocation, 3T3-L1 adipocytes were stimulated with insulin for 30 min. The plasma membrane and cytoplasmic proteins were extracted and fractionated using the ProteoJETTM Membrane Protein Extraction kit (Fermentas, Shenzhen, China) according to the manufacturer's recommended protocol. For determination of NF-κB p65 nuclear translocation, the cytosolic and nuclear proteins from cells were separated and isolated by Nuclear-Cytosol Extraction Kit (Applygen Technologies Inc, Beijing, China) following the manufacturer's instructions.

Fifty micrograms of total, membranous, cytoplasmic or nuclear protein was electrophoresed on a polyarcrylamide gel and transferred to polyvinylidene difluoride membranes by electroblotting. The membranes were blocked for 2 h at RT with 5% skim milk in PBS containing 0.1% Tween-20 and then probed overnight at 4 °C with primary antibodies including anti-GLUT4 and anti-PPAR-γ (Millipore, Billerica, MA), anti-IκB-α, anti-p-Tyr-IRS-1, anti-IRS-1, anti-Akt, anti-TNF-α, anti-Lamin B1, anti-caveolin-1 and anti-β-actin (Santa Cruz, CA), anti-p-Akt (Cell Signaling Technology, Beverly, MA) and anti-NF-κB p65 (Epitomics, Burlingame, CA), followed by corresponding horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibody (Pierce, Rockford, IL) at RT for 1 h. Immunoreactive bands were visualized using the enhanced chemiluminescence kit (Pierce, Rockford, IL) and the Kodak Image Station 4000 MM (East-man Kodak Co., Rochester, NY).

2.5. Flow cytometry

After stimulation, 3T3-L1 adipocytes (2×10^5 cells/well) were incubated for 1 h at 4 °C with an antibody specific to murine TNF-α (Abcam, Cambridge, MA), followed by a 45 min-incubation at 4 °C with fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Surface expression of tmTNF-α was analyzed on a FACS Calibur 440 E flow cytometer (Becton Dickinson, San Jose, CA).

2.6. ELISA for adipokines

Commercial ELISA kits were used to detect sTNF-α, IL-6, MCP-1 (eBioscience, San Diego, CA) and adiponectin (LINCO, St. Charles, MO) in the supernatants of cultured 3T3-L1 adipocytes according to the manufacturers' protocols.