Effect of short hairpin RNA-mediated adiponectin/Acrp30 down-regulation on insulin signaling and glucose uptake in the 3T3-L1 adipocytes

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ABSTRACT. Adiponectin is a polypeptide hormone that is secreted by adipocytes with insulin-sensitizing and anti-inflammatory properties. The current study was to further investigate the role of adiponectin on glucose uptake and its underlying mechanism by down-regulation of adiponectin in 3T3-L1 adipocytes. Transfection of short hairpin RNA (shRNA)-vector significantly decreased adiponectin mRNA expression and its protein level in the cells. The down-regulation of adiponectin markedly reduced the cellular glucose uptake rate and increased intracellular triglyceride content. To study the mechanism of the physiologic action of adiponectin, several key regulatory factors in insulin signaling pathway were examined. The mRNA expression of insulin receptor substrate (IRS)-1 in both basal and insulin-stimulated states were down-regulated in the transfected cells (72% and 52% of controls, respectively), and the insulin-stimulated IRS-1 tyrosine phosphorylation was also significantly decreased. Adiponectin-deficient cells showed marked down-regulations of peroxisome proliferator-activated receptor α, glucose transporter (GLUT)-1, GLUT-4, hormone-sensitive lipase (HSL), and adipose triglyceride lipase. These results thus demonstrated that transfection of shRNA-vector effectively reduced the expression of adiponectin in 3T3-L1 adipocytes accompanied with a significant decrease in cellular glucose uptake rate and an increase in intracellular triglyceride content. Our data also suggested that adiponectin deficiency impairs insulin action in vitro probably through the IRS-1 pathway, and increase intracellular fat accumulation partially through HSL down-regulation. (J. Endocrinol. Invest. 33: 96-102, 2010) ©2010, Editrice Kurtis

INTRODUCTION

Adiponectin is a polypeptide hormone that is secreted by adipocytes with insulin-sensitizing and anti-inflammatory properties. The current study was to further investigate the role of adiponectin on glucose uptake and its underlying mechanism by down-regulation of adiponectin in 3T3-L1 adipocytes. Transfection of short hairpin RNA (shRNA)-vector significantly decreased adiponectin mRNA expression and its protein level in the cells. The down-regulation of adiponectin markedly reduced the cellular glucose uptake rate and increased intracellular triglyceride content. To study the mechanism of the physiologic action of adiponectin, several key regulatory factors in insulin signaling pathway were examined. The mRNA expression of insulin receptor substrate (IRS)-1 in both basal and insulin-stimulated states were down-regulated in the transfected cells (72% and 52% of controls, respectively), and the insulin-stimulated IRS-1 tyrosine phosphorylation was also significantly decreased. Adiponectin-deficient cells showed marked down-regulations of peroxisome proliferator-activated receptor α, glucose transporter (GLUT)-1, GLUT-4, hormone-sensitive lipase (HSL), and adipose triglyceride lipase. These results thus demonstrated that transfection of shRNA-vector effectively reduced the expression of adiponectin in 3T3-L1 adipocytes accompanied with a significant decrease in cellular glucose uptake rate and an increase in intracellular triglyceride content. Our data also suggested that adiponectin deficiency impairs insulin action in vitro probably through the IRS-1 pathway, and increase intracellular fat accumulation partially through HSL down-regulation. (J. Endocrinol. Invest. 33: 96-102, 2010) ©2010, Editrice Kurtis

Key-words: 3T3-L1 adipocytes, adiponectin, glucose turnover, insulin signaling, RNA interference.

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Accepted June 29, 2009.
First published online July 28, 2009.
ed RNA interference (RNAi) technique that utilizes short hairpin RNA (shRNA) to substantially and stably knock down adiponectin expression in 3T3-L1 adipocytes. The in vitro approach allows us to specifically investigate the mechanism of adiponectin actions without the systematic influences in animal model. In the study we examined the effects of adiponectin/Acrp30 down-regulation on cellular glucose uptake, triglyceride content and the key molecules in insulin signaling including IRS-1, peroxisome proliferator-activated receptor (PPAR), glucose transporter (GLUT)1, GLUT4, hormone-sensitive lipase (HSL), and adipose triglyceride lipase (ATGL).

MATERIALS AND METHODS

Construction of adiponectin shRNA expression plasmids

Three shRNA sequences for target gene were designed and synthesized. The sequences of the 3 cDNA fragments (sense strands) were as follow: 1) 5'-AAGGATACGGTACCAACTGT-3'; 2) 5'-AAGGATACGGTACCAACTGT-3'; 3) 5'-AATGGACTCTATGCAGATAAC-3'. These oligonucleotides were annealed and inserted into pSilencer1.0-U6 vector (Ambion,USA) to create pSilencer1.0-U6-Acrp30-1, pSilencer1.0-U6-Acrp30-2 and pSilencer1.0-U6-Acrp30-3, respectively. An insert control of Green Fluorescent Protein (GFP) shRNA oligonucleotide was used as the negative control (pSilencer1.0-U6-GFP). Recombinant plasmids were verified by Ntot and KpnI digestion analysis and DNA sequencing.

Construction of shRNA-expressing adenoviruses

The plasmids (pSilencer1.0-U6-Acrp30 and pSilencer1.0-U6-GFP) and the shuttle vectors (pShuttle) were digested with Pme I and ligated. The shuttle plasmids (pShuttle-U6-Acrp30) and pShuttle-GFP) were linearized by Pme I and transformed into XL10-Gold cells. Miniprep plasmid DNA was made by a standard alkaline lysis procedure. The resulting adenoviral DNA (pAd-U6-Acrp30) and pAd-U6-GFP) were verified by PacI digestion analysis and DNA sequencing then transformed into XL10-Gold for large scale amplification.

3T3-L1 cell culture

3T3-L1 preadipocytes were cultured and induced for differentiation as previously described (15). In brief, 3T3-L1 preadipocytes were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 25 mM glucose and 10% calf serum in 5% CO2 at 37 C. After complete confluence in about 2 days, cell differentiation was induced by incubating the cells in DMEM containing 25 mM glucose, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone, 10 μg/ml insulin, and 10% fetal bovine serum (FBS) for 48 h. Then the medium was replaced and the cells were incubated with DMEM containing 25 mM glucose, 10 μg/ml insulin, and 10% FBS for another 48 h. The cell culture was maintained in DMEM containing 25 mM glucose and 10% FBS, and the medium was changed every 2 days until 90% of cells exhibited the adipocyte phenotype.

RNA interference

The pAd-U6 vectors (pAd-U6-Acrp30 and pAd-U6-GFP) were transfected into the differentiated 3T3-L1 adipocytes by using Lipofectamine™2000 (Invitrogen). The transfection was performed according to the manufacturer’s protocol. 3T3-L1 adipocyte transfected pAd-U6-GFP was used as a negative control, untreated 3T3-L1 adipocytes as the blank control and 3T3-L1 preadipocyte as the background control. Before each experiment, cells were serum starved for 3-4 h in DMEM containing 0.1% BSA, and then incubated with or without 0.6 mM insulin at 37 C for 12 h (16). Three days (72 h) following transfection, cells and supernatants were collected for RT-PCR and enzyme-linked immunosorbent assay (ELISA) analyses.

Measurement of glucose uptake rate

After the transfection for 72 h, adipocytes in 24 well plate were washed 3 times with preheated phosphate buffer saline (PBS) buffer, incubated with 0.5 ml PBS buffer containing 2-Deoxy-[3H]-D-glucose (1 μCi/ml) for 10 min at 37 C, washed again 3 times with pre-cooled PBS buffer, incubated with 4 ml NaOH (0.2 mmol/l for 4 h, then neutralized with 0.2 ml HCl (0.4 mol/l). The rate of disintegration per minute were counted as the uptake rates of 2-deoxy-[3H]-D-glucose (GUR).

Measurement of triglyceride content in 3T3-L1 adipocytes

The cellular triglyceride content of 3T3-L1 adipocytes grown in 24-well plates was measured with a colorimetric assay. For Oil Red O staining, 0.3% Oil Red O in 100% isopropanol was used. 3T3-L1 adipocytes were fixed in 10% formaldehyde for 30 min, incubated with Oil Red O for 15 min, washed 3 times with 60% isopropanol, eluted Oil Red O by adding 100% isopropanol for 10 min and measured OD at 490 nm.

RT-PCR analysis

Total RNA was prepared from 3T3-L1 adipocytes with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Reverse transcription was performed on 1 μg total RNA using RNase M-MLV (RNase H, TaKaRa Bio Co., Ltd, Dalian) and Oligo (dT)18 Primer (TaKaRa Bio Bio Co., Ltd, Dalian). The PCR reaction mixture in a total volume of 2 μl of the first strand cDNA, 2 μl of 10 pmol specific primers, 4 μl of 2.5 mM dNTP, 5 L 10×buffer, 2.5 U Taq DNA polymerase, 3 μl of 2.5 mM MgCl2, dH2O up to 50 μl. Samples were incubated for an initial denaturation at 94 C for 3 min, followed by 30-45 cycles (depend on different genes) of 94 C for 30 sec, 55 C for 30 sec, 72 C for 50 sec, and finally, one cycle of 72 C for 5 min. The intensities of PCR bands were quantified with a Bio Imaging System Densitometer (Bio-Rad). The sequences, product lengths, and annealing temperatures of the primers are shown in Table 1.

ELISA

ELISA for the measurement of adiponectin released in the culture medium from the 3T3-L1 cells was carried out in 96-well ELISA plates according to the manufacturer’s instructions (Phoenix Pharmaceuticals, Belmont, CA). All ELISA measurements were done in triplicate.

IRS-1 tyrosine phosphorylation assay and intracellular GLUT-4 protein assays

Cells were harvested from the culture plates 72 h after transfection, then incubated with or without 100 nM insulin at 37 C for