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Lipid synthesis is promoted by hypoxic adipocyte-derived exosomes in 3T3-L1 cells

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ABSTRACT

Hypoxia occurs within adipose tissues as a result of adipocyte hypertrophy and is associated with adipocyte dysfunction in obesity. Here, we examined whether hypoxia affects the characteristics of adipocyte-derived exosomes. Exosomes are nanovesicles secreted from most cell types as an information carrier between donor and recipient cells, containing a variety of proteins as well as genetic materials. Cultured differentiated 3T3-L1 adipocytes were exposed to hypoxic conditions and the protein content of the exosomes produced from these cells was compared by quantitative proteomic analysis. A total of 231 proteins were identified in the adipocyte-derived exosomes. Some of these proteins showed altered expression levels under hypoxic conditions. These results were confirmed by immunoblot analysis. Especially, hypoxic adipocyte-released exosomes were enriched in enzymes related to *de novo* lipogenesis such as acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, and fatty acid synthase (FASN). The total amount of proteins secreted from exosomes increased by 3–4-fold under hypoxic conditions. Moreover, hypoxia-derived exosomes promoted lipid accumulation in recipient 3T3-L1 adipocytes, compared with those produced under normoxic conditions. FASN levels were increased in undifferentiated 3T3-L1 cells treated with FASN-containing hypoxic adipocytes-derived exosomes. This is a study to characterize the proteomic profiles of adipocyte-derived exosomes. Exosomal proteins derived from hypoxic adipocytes may affect lipogenic activity in neighboring preadipocytes and adipocytes.
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1. Introduction

Adipose tissues store excess energy in the form of lipids [1,2]. The tissues are the largest energy reserve in mammals and are capable of accommodating prolonged nutrient excess by altering their mass. However, abnormal or excess accumulation of lipids in adipose tissues causes obesity, which may impair health [3–5]. Adipose tissue expansion occurs when adipocyte numbers and size increase, which is known as hyperplasia and hypertrophy, respectively [6]. Limiting adipocyte hyperplasia leads to lipid accumulation in existing adipocytes, resulting in hypertrophy. Uptake of exogenous lipids or synthesis of endogenous lipids in the cytosol causes hypertrophy. Smaller adipocytes may be more likely to synthesize fatty acids endogenously (*de novo* lipogenesis) to begin the lipids accumulation process, while uptake of exogenous fatty acids is more predominant in developing cells [7].

De novo lipogenesis [8] is the process in which non-lipid precursors are converted to fatty acids, and requires acetyl-CoA, which is generated during various metabolic processes. Acetyl-CoA provides the carbon atoms necessary for fatty acid synthesis. It is converted to malonyl-CoA, and the rate-limiting steps in *de novo* lipogenesis are catalyzed mainly by acetyl-CoA carboxylase (ACC). Successive

Abbreviations: ACN, acetonitrile; ACC, acetyl-CoA carboxylase; FASN, fatty acid synthase; FBS, fetal bovine serum; G6PD, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-phosphate dehydrogenase; Hsc70, heat shock cognate 71 kDa protein; Hsp, heat shock protein; Hsp72, heat shock 71 kDa protein 1A; HIF-1α, hypoxia-inducible factor-1α; WT, wild-type.

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malonyl-CoA molecules, which serve as a two-carbon donor, are added to acetyl-CoA by the multi-functional enzyme complex, fatty acid synthase (FASN). Glucose-6-phosphate dehydrogenase (G6PD) is a key enzyme that supplies the cellular NADPH required for lipid biosynthesis.

Adipocytes have a limited capacity to accumulate lipid droplets. When adipocytes suffer from lipid overload, hypoxia develops; the reduction in oxygen tension is directly linked to adipocyte dysfunction. To avoid lipid overload and the associated cellular stress in adipose tissues, expression of enzymes related to *de novo* lipogenesis is reduced [9]. Additionally, adipocytes do not increase in size in a synchronized fashion [10]. Small adipocytes and preadipocytes can act as reservoirs by increasing their storage capacity when larger adipocytes no longer accommodate increased lipid storage. However, how adipocytes without lipid overload are activated to store excess energy remains unknown. Adipocytes communicate with each other and with other tissues [11], but the types of communication between stressed larger adipocytes under hypoxic stress and non-stressed, less hypoxic adipocytes are unknown. Three types of signals are known to control communication between adipocytes [11]: cell-to-cell contact, soluble factors, and exosomes.

Exosomes are small 50–150 μm membrane vesicles secreted from most cell types [12]; they play an important role as information carriers between donor and recipient cells. Exosomes contain a wide variety of cytosolic contents as well as membranous components from donor cells, including genetic materials, lipids, and proteins, which determine the types of information carried [13,14]. Exosome content is thought to reflect the conditions surrounding the donor cells [15]. Exosomes could fuse with and transfer their internal contents into the cytosol of recipient cells [14]. Upon interacting with exosomes and receiving the internal contents, recipient cells undergo morphological and physiological changes, including cancer metastasis, angiogenesis, and cell differentiation [16–19]. Adipocytes also secrete exosomes [20]; however, the characteristics of adipocyte-derived exosomes are poorly understood, particularly under pathological conditions.

In this study, we first conducted quantitative proteomic analysis in 3T3-L1 adipocyte-derived exosomes. We demonstrated that multiple enzymes related to *de novo* lipogenesis were enriched in exosomes secreted under hypoxic conditions. These exosomes may promote lipid accumulation by transferring lipogenic enzymes into recipient cells.

2. Materials and methods

2.1. Reagents, cell lines, and animals

Detailed material information can be found in the data supplement.

2.2. Exosome purification

Donor cells (3T3-L1 cells or HEK 293T cells) were cultured in DMEM (4500 mg/L glucose) supplemented with 10% exosomes-depleted fetal bovine serum (FBS). Exosomes were depleted of FBS by 12 h ultracentrifugation at 100,000*g*, 4 °C. Exosomes were prepared from cell supernatants using sequential centrifugation and filtration steps. Briefly, cell supernatants were diluted in an equal volume of phosphate-buffered saline (PBS) and centrifuged for 30 min at 2000*g*, 4 °C. The supernatants were centrifuged for 60 min at 10,000*g*, 4 °C. Next, the supernatants were ultracentrifuged for 3 h at 100,000g, 4 °C. Pellets were resuspended in a large volume of PBS and the suspension was filtered through a 0.22-μm filter. Exosomes were pelleted by 3 h of ultracentrifugation at 100,000*g*, 4 °C. Total amount of exosomes was determined by using the BCA assay (Thermo Scientific, Rockford, IL, USA). Purified exosomes were resuspended in PBS and stored at −80 °C until use.

2.3. Electron microscopy analysis

PBS-resuspended exosomes were deposited onto formvar/carbon-coated EM grids (EMJapan Co., Ltd., Tokyo, Japan). Membranes were allowed to absorb for 10 min in a dry environment; excess liquid was removed gently using absorbent paper. Exosome-coated grids were stained with 1% uranyl acetate and the preparations were examined under a transmission electron microscope (TEM; H-7500, JEOL Ltd., Tokyo, Japan).

2.4. Proteomic analysis of purified exosomes

Exosomes purified from supernatants of 3T3-L1 adipocytes under normoxic (20% O_2) or hypoxic (1% O_2) for 48 h were solubilized in 0.8% RapiGest SF (Waters, Milford, MA, USA) and lysed by 3 freeze–thaw cycles in liquid nitrogen under sonication. Next, 60 μg of exosomal proteins were reduced, alkylated, digested with trypsin, and labeled with 2-plex iTRAQ reagents (AB Sciex, Framingham, MA, USA) according to the manufacturer's instructions with minor modifications. After the labeling reaction (114, Normoxia; 116, hypoxia), the 2 samples were pooled and 10 μL of 20% (v/v) trifluoroacetic acid was added to cleave the RapiGest. Samples were vortexed, incubated at 37 °C for 1 h and centrifuged. Supernatants were purified using a cation exchange column (AB Sciex) using standard procedures, as previously described [21]. Briefly, mobile phase A contained 98% water (2% acetonitrile (ACN), 0.1% formic acid) and mobile phase B contained 70% ACN (0.1% formic acid, 30% water). The column effluent was introduced into a spray chamber through a tapered stainless steel emitter and directly electrosprayed into the QSTAR System ion trap mass spectrometer in positive mode for nanoESI-tandem mass spectrometry (MS/MS) analysis. Each sample was run for 150 min. Protein identification was performed using Analyst QS Software 2.0 (AB Sciex) in positive-ion mode. Both data sets were processed using ProteinPilot Software 2.0.1 with the Paragon™ search algorithm (AB Sciex). MS/MS data were searched against the NCBI database using a *Mus musculus* taxonomy filter. The minimum threshold for protein identification was set at a protein score of 0.47, corresponding to a confidence level >66% and 1% false discovery rate.

2.5. Immunoblot analysis

Detailed material information can be found in the data supplement.

2.6. Exosomes taken up by 3T3-L1 cells

For red fluorescent labeling of cells, we incubated HEK 293T cells with PKH26 (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. 3T3-L1 preadipocytes were cultured in media containing PKH26 exosomes.

2.7. Statistical analysis

All data are presented as means ± SD. Data were analyzed using paired Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Serum exosomes are increased in obese animals

To examine whether obesity affects serum exosomes, serum exosomes from leptin-deficient (ob/ob) obesity mice and wild-type

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