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Alpha-lipoic acid attenuates adipocyte differentiation and lipid accumulation in 3T3-L1 cells via AMPK-dependent autophagy

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ABSTRACT

Aims: During the adipocyte differentiation, some intracellular organelles are degraded and instead lipid droplets are gradually accumulated in the cytoplasm for energy storage. Autophagy, a self-eating process, has been implicated in the removal of intracellular components in adipogenesis, but its mechanism is poorly understood. In this work we examined how α-lipoic acid modulates the autophagic process during the adipocyte differentiation.
Main methods: 3T3-L1 pre-adipocytes were differentiated in the medium containing insulin, dexamethasone, and 1-methyl-3-isobutylxanthine. Lipid contents in adipocytes were determined by Oil-Red O staining. Autophagy was evaluated by Western blotting, accumulation of acidic vacuoles in cells.
Key findings: We observed that formation of LC3-II, an indicative marker for autophagy, was greatly down-regulated at the beginning stage of differentiation, but it was gradually increased with respect to earlier differentiation time. In addition, ATG5-12 conjugates were similarly produced, and acidic autophagic vacuoles were greatly elevated at the earlier stages of differentiation. Furthermore, α-lipoic acid deteriorated the intracellular accumulation of lipid droplets by blocking the production of acidic autophagic vacuoles, LC3-II, and other autophagy-related proteins during the adipocyte differentiation and influenced expression of adipocyte-stimulating factors. It also specifically suppressed activation of AMPK, an essential modulator for autophagy, at the earlier step of adipocyte differentiation.
Significance: These data suggest that α-lipoic acid significantly attenuates adipocyte differentiation via the direct modulation of intracellular degradation process and consequently decrease intracellular fat deposit of adipocytes.

Introduction

Adipogenesis is an overall differentiation process, stimulating fibroblast-like pre-adipocytes to become terminally differentiated mature adipocytes. They generally have a distinctive cellular structure in which the cytoplasmic space is occupied by lipid droplets. Pre-adipocytes undergo differentiation in response to adipogenic signals and activate two major transcriptional factors such as C/EBPs and PPARγ, sequentially turn on enzymes necessary for lipid biosynthesis (Rosen and Spiegelman, 2000; Rosen and MacDougald, 2006). The excessive accumulation of terminally differentiated adipocytes in the body generally causes obesity, associating with high accumulation of triglycerides and other energy materials and general impairment of catabolic pathways. Therefore, understanding of the mechanistic basis of adipocyte differentiation is very crucial for controlling obesity-dependent diseases.

The adipocyte differentiation process absolutely requires removal of intracellular components such as mitochondria, peroxisome and others in order to replace them with lipid droplets. Although mechanism about the removal of these intracellular components during adipogenesis is not clearly elucidated, so far autophagy is one of the best well-characterized processes to massively degrade intracellular proteins and organelles. Autophagy, a self-eating process, requires formation of double-membrane autophagosomes in which intracellular damaged proteins or organelles were engulfed and degraded by lysosomal proteases after being fused with lysosomes (Mizushima et al., 2008). Therefore, this process is involved in many cellular stress responses such as cell adaptation under nutrient deprivation, cell death, removal of damaged proteins or organelles or cell survival in oxidative stress. In addition

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to these responses, autophagy has been implicated to play a possible role in the adipocyte differentiation because of the massive removal capacity of intracellular components (Dong and Czaja, 2011; Goldman et al., 2011; Zhang et al., 2012).

Indeed, there are some recent papers describing the connection between autophagy and adipogenesis. The mice exhibiting deficiency of autophagy genes such as Atg5 or Atg7 showed a large decrease of lipid accumulation and impaired adipogenesis, determining overall body mass of adipose-tissues in mice (Zhang et al., 2009; Singh et al., 2009a). The similar impairment of adipocyte differentiation was also observed in 3T3-L1 preadipocytes or primary mouse embryonic fibroblast cells in which Atg5 or Atg7 was deleted or the autophagic process was blocked by chemical inhibitors (Baerga et al., 2009). These results clearly suggest that autophagy is related to the removal process of intracellular components during adipogenesis, but the mechanistic basis on how to initiate autophagy from the signaling of adipocyte differentiation and how these two processes to be mutually regulated remains to be solved.

AMP-activated protein kinase (AMPK), a principal metabolic sensor to balance the cellular nutrient level, plays a key role in both autophagy and lipid metabolism. At a low energy status including starvation, AMPK can be activated by LKB1 or by increased AMP/ATP ratio, which inhibits mTOR, a negative regulator for autophagy. Consequentially, this process promotes autophagy induction (Gwinn et al., 2008; Inoki et al., 2003). In addition, the activated AMPK directly modulates activity of ULK1 by phosphorylation to induce autophagy together with other proteins (Egan et al., 2011; Kim et al., 2011). Furthermore, AMPK has been implicated in regulating lipid metabolism. Its activation by fasting or hypoglycemic drugs stimulates lipolysis and leads to increase the cellular energy level (Daval et al., 2006; Minokoshi et al., 2002).

In the current study, we suggest that α-lipoic acid, an anti-oxidant and anti-obesity drug, directly inhibits adipocyte differentiation by modulating expression or activity of proteins necessary for the autophagic process in 3T3-L1 preadipocytes and eventually decreases lipid accumulation in adipocytes. These results partially support some previous observations in which α-lipoic acid can inhibit the activity of AMPK and, in turn, deteriorate fatty acid biosynthesis (Kim et al., 2004; Koh et al., 2011).

Materials and methods

Reagents

Alpha-lipoic acid, insulin, 1-methyl-3-isobutylxanthine, dexamethasone, Oil-Red O dye, and monodansylcadaverine (MDC) were purchased from Sigma (St. Louis, MO). Acridine orange dye was obtained from Invitrogen (Molecular Probes, Carlsbad, CA). Antibodies used in the study were as follows: p-mTOR and mTOR (1:1000), p-AMPK and AMPK (1:1000), p-MAPK and MAPK (1:1000) from Cell Signaling (Beverly, MA), LC3 (1:3000) from Abcam, and Beclin-1 (1:1000), and Atg5 (1:500) from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies against rabbit (1:3000), mouse (1:3000), and goat (1:3000) immunoglobulins were purchased from Bio-Rad (Hercules, CA).

Adipocyte differentiation

3T3-L1 preadipocytes were cultured and maintained in 1× Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO, Carlsbad, CA) and antibiotics (500 μg/mL penicillin and 500 μg/mL streptomycin; GIBCO) (maintenance medium) at 37 °C in a humidified atmosphere with 5% CO_2. For adipocyte differentiation, 100% confluent 3T3-L1 cells after 2-day further incubation for the complete cell cycle arrest were incubated to begin clonal expansion in MDI differentiation medium (maintenance medium supplemented with 160 nM insulin, 250 nM dexamethasone, and 0.5 mM 1-methyl-3-isobutylxanthine) (day 0). Two days later, the cells were further incubated in maintenance medium supplemented with 160 nM insulin, and subsequently incubated in the maintenance medium after another 2 days later. After then, the half of cell medium was replaced by the fresh maintenance medium every 2 days until the cells were completely differentiated.

Acridine orange dye staining

Cell-staining with acridine orange dye was performed as previously described (Ha et al., 2012). Acridine orange (AO) dye was added to the cells at a final concentration of 1 μg/mL and incubated for 15 min at room temperature. Red (acidic vacuoles) and green (cytoplasm) fluorescent images were obtained under a fluorescent microscope (Leica, Wetzlar, Germany) using color filters for TRITC and FITC, respectively. Then, overlapped images were processed and quantified using Image J software.

Monodansylcadaverine (MDC) staining

MDC staining was performed as previously described (Ha et al., 2012). 3T3-L1 cells (3×10^5 cells in 3 mL of growth medium) were seeded in 6-well plates with sterile cover slips. Following the desired stimuli for induction or inhibition of autophagy, cells were incubated with 50 μM MDC in growth medium for 30 min at 37 °C. Cells were washed once with 1× PBS, and MDC-stained cells were detected under a fluorescent microscope (Leica).

Western blot analysis

3T3-L1 cells were collected at the indicated days, washed twice with ice-cold 1× PBS, and lysed in the RIPA buffer (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (1 mM phenymethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin) on ice for 1 h. Cell lysates were centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was then collected for SDS-PAGE analysis. Protein concentration was determined using the Bio-Rad protein assay kit. Total proteins (30 μg) were separated on a 10% SDS-PAGE gel unless indicated otherwise and transferred to a nitrocellulose membrane using a semi-dry transfer system (Bio-Rad) for 40 min at 15 V. The membrane was blocked for 1 h at room temperature in the 5% skim milk/TBST (50 mM Tris–HCl, pH 7.4, 0.1 M NaCl and 0.1% tween-20) solution. After incubation with primary antibodies overnight at 4 °C in 5% skim milk/TBST, the membrane was then washed three times in TBST for 10 min and incubated with secondary antibodies for 1 h (1:3000 in 5% skim milk/TBST). The membrane was washed three times in TBST for 10 min. Specific proteins were visualized using ECL detection system (Thermo Scientific, Pittsburgh, PA).

Oil Red O staining and quantification

Cells were washed twice with 1× PBS, fixed in 3.7% formaldehyde for 10 min, and then washed three times with cold water. Cells were stained in the Oil Red O working solution (6:4, 0.6% Oil Red O dye in isopropanol: water) for 30 min at 25 °C and washed three times with water. Staining was visualized by bright-field microscopy, and Oil Red O dyes extracted from the cells in isopropanol solution containing 4% Nonidet P-40 were quantified at a wavelength of 520 nm.

Statistics

Data were represented as mean of at least three independent experiments. Difference between two groups was assessed by *Student-t* test. $p < 0.05$ was considered as significance.