Biochemical and Biophysical Research Communications 478 (2016) 12–17

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

RKIP phosphorylation–dependent ERK1 activation stimulates adipogenic lipid accumulation in 3T3-L1 preadipocytes overexpressing LC3

Jong Ryeal Hahm [a, c], Mahmoud Ahmed [b, c], Deok Ryong Kim [b, c, *]

[a] Department of Internal Medicine, Gyeongsang National University School of Medicine, JinJu, 527-27, Republic of Korea
[b] Department of Biochemistry and Convergence Medical Science, Gyeongsang National University School of Medicine, JinJu, 527-27, Republic of Korea
[c] Institute of Health Sciences, Gyeongsang National University School of Medicine, JinJu, 527-27, Republic of Korea

ARTICLE INFO

Article history:
Received 19 July 2016
Accepted 24 July 2016
Available online 25 July 2016

Keywords:
Adipogenesis
RKIP
ERK1
LC3
Lipid droplets
3T3-L1

ABSTRACT

3T3-L1 preadipocytes undergo adipogenesis in response to treatment with dexamethaxone, 1-methyl-3-isobutylxanthine, and insulin (DMI) through activation of several adipogenic transcription factors. Many autophagy-related proteins are also highly activated in the earlier stages of adipogenesis, and the LC3 conjugation system is required for formation of lipid droplets. Here, we investigated the effect of overexpression of green fluorescent protein (GFP)-LC3 fusion protein on adipogenesis. Overexpression of GFP-LC3 in 3T3-L1 preadipocytes using poly-L-lysine-assisted adenoviral GFP-LC3 transduction was sufficient to produce intracellular lipid droplets. Indeed, GFP-LC3 overexpression stimulated expression of some adipogenic transcription factors (e.g., C/EBPα or β, PPARγ, SREBP2). In particular, SREBP2 was highly activated in preadipocytes transfected with adenoviral GFP-LC3. Also, phosphorylation of Raf kinase inhibitory protein (RKIP) at serine 153, consequently stimulating extracellular-signal regulated kinase (ERK)1 activity, was significantly increased during adipogenesis induced by either poly-L-lysine-assisted adenoviral GFP-LC3 transduction or culture in the presence of dexamethasone, 1-methyl-3-isobutylxanthine, and insulin. Furthermore, RKIP knockdown promoted ERK1 and PPARγ activation, and significantly increased the intracellular accumulation of triacylglycerides in DMI-induced adipogenesis. In conclusion, GFP-LC3 overexpression in 3T3-L1 preadipocytes stimulates adipocyte differentiation via direct modulation of RKIP-dependent ERK1 activity.

1. Introduction

3T3-L1 preadipocytes initiate adipogenic differentiation *in vitro* in medium supplemented with 250 nM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine, and 160 nM insulin (DMI) by activating major adipogenic transcription factors, such as C/EBPs and PPARγ, and subsequently upregulating several enzymes that are involved in lipid biosynthesis [1–4]. Through these adipogenic differentiation processes, lipid droplets accumulate extensively in the cytoplasm of differentiated adipocytes. *In vitro* adipogenesis using 3T3-L1 cells is widely used to study the molecular mechanism of adipocyte differentiation and to identify target molecules for adipocyte-associated metabolic diseases.

Many transcription factors are specifically expressed and activated during adipocyte differentiation. The CCAAT/enhancer-binding proteins β and δ (C/EBPβ and C/EBPδ), which are specifically expressed at the earlier stage of adipogenesis, trigger the enormous mitotic clonal expansion to initiate differentiation of growth-arrested 3T3-L1 preadipocytes [3,4]. In particular, C/EBPβ transcriptionally activates C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ), the master regulators of adipocyte differentiation. C/EBPα and PPARγ subsequently induce expression of many adipocyte genes necessary for the development of adipose tissue. C/EBPβ is also regulated by numerous kinases, including PKA, mitogen-activated protein kinase (MAPK), and glycogen kinase 3β [5,6]. In particular, extracellular-signal regulated kinase (ERK)-dependent phosphorylation of C/EBPβ in 3T3-L1 cells plays a critical role in adipogenesis [4,7], and many previous reports suggest that the ERK pathway might be involved in regulation of

* Corresponding author. Department of Biochemistry and Convergence Medical Science, Gyeongsang National University School of Medicine, JinJu, 527-27, Republic of Korea.
E-mail address: drkim@gnu.ac.kr (D.R. Kim).

http://dx.doi.org/10.1016/j.bbrc.2016.07.107
0006-291X/

adipocyte differentiation [8–10].

Raf kinase inhibitory protein (RKIP) inhibits the intracellular ERK pathway, a major mitogen-induced signaling pathway, through a direct association with RAF, an upstream kinase of ERK [11,12]. In addition to modulating ERK signaling, RKIP also acts as a key mediator in many other intracellular signaling pathways, including the NF-κB, GRK2, or glycogen synthase kinase 3β (GSK3β) pathways [13–15]. As we showed in a recent study, RKIP negatively regulates the Notch pathway, consequently suppressing epithelial mesenchymal transition and metastasis in cancer cells [16]. In addition, phosphorylation of RKIP at serine 153 by protein kinase C induces release of Raf kinase from the inactive RKIP/Raf complexes and results in activation of ERK signaling [14]. Therefore, control of RKIP activation and expression might be critical in ERK-dependent adipocyte growth and differentiation.

The microtubule-associated protein 1A/1B light chain 3 (LC3), a key regulator of the self-degradative process of autophagy, is covalently linked to membrane-anchored phosphatidylethanolamine (PE) through consecutive conjugation reactions of autophagy-related proteins, including Atg4B, Atg7, Atg3, Atg5, and Atg16L [17–19]. PE-conjugated LC3 (LC3-II) plays a critical role in formation of autophagosomes and sequestration of cellular components into the autophagosomal lumen via specific protein-protein interactions [20]. Autophagy is also functionally associated with cell development and differentiation. In particular, autophagy is directly involved in the removal of intracellular components during adipogenesis [21–24]. In addition, the LC3 conjugation system has been suggested to play a role in formation of lipid droplets, a storage form of triacylglycerols derived from the endoplasmic reticulum (ER) and released into the cytosol in eukaryotic cells, although the mechanistic basis of lipid-droplet formation is unclear [25].

In the present study, we noted that overexpression of green fluorescent protein (GFP)-LC3 was sufficient for adipocyte differentiation in 3T3-L1 preadipocytes transfected with adenoviral GFP-LC3 vectors. Furthermore, ERK1 activity was significantly upregulated in a RKIP phosphorylation-dependent manner during adipogenesis.

2. Materials and methods

2.1. Reagents

Insulin, 1-methyl-3-isobutylxanthine, dexamethasone, poly-L-lysine hydrobromide (molecular weight 30,000–70,000), and Oil Red O were purchased from Sigma (St. Louis, MO). Antibodies to the following proteins were used in this study: C/EBPα, C/EBPβ, SREBP1, SREBP2, PPARγ, *p*-AKT and AKT (1:1000), *p*-ERK and ERK (1:1000) from Cell Signaling Technology (Danvers, MA); LC3 (1:1000) from Abcam (Cambridge, UK); and β-actin (1:3000) from Sigma. Antibodies to *p*-RKIP and RKIP (1:1000) and secondary antibodies against rabbit (1:3000), and mouse (1:3000) immunoglobulins were purchased from Bio-Rad (Hercules, CA).

2.2. Construction of recombinant adenoviral vectors

cDNA encoding human LC3B or GFP that was amplified by PCR was subcloned into the pAdCMV/V5–DEST gateway cloning vector (Invitrogen, Carlsbad, CA), resulting in pAdCMV/V5–DEST-GFP-LC3B (adenoviral GFP-LC3 fusion vector, Ad-GFP-LC3) or pAdCMV/V5–DEST-GFP (control, Ad-GFP). Adenoviral shRKIP expression vector (Ad-shRKIP) and GFP control vector (Ad-GFP) have been described previously [16]. For adenoviral infection, cells were plated in 6-well plates at a density of 1×10^5 cells/mL, infected with recombinant adenoviruses at a multiplicity of infection (MOI) of 100, and then further incubated at 37 °C.

2.3. Adipocyte differentiation and adenoviral transduction

3T3-L1 preadipocytes were cultured and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum (Gibco, Carlsbad, CA) at 37 °C in a humidified atmosphere of 5% CO_2. Three different methods for adipocyte differentiation were used in this work. First, 3T3-L1 preadipocytes were incubated in DMEM containing 250 nM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine, and 160 nM insulin (MDI) and, after two days, further incubated in DMEM supplemented with 160 nM insulin for two days. Cells were then cultivated in DMEM supplemented with 10% FBS until cells were completely differentiated. Second, 90% confluent 3T3-L1 preadipocytes were infected with Ad-GFP or Ad-GFP-LC3 particles in serum-free medium containing 1 μg/mL poly-L-lysine as previously described [26], and after 24 h, cells were continuously incubated in DMEM containing 10% FBS, with replacement of medium with fresh medium every two days until cells were differentiated. Lastly, the 3T3-L1 cells transfected with Ad-GFP or Ad-shRKIP were incubated for 24 h, when cells reached confluence. Confluent 3T3-L1 cells were further incubated for 2 days and then treated with MDI. Two days later, the cells were incubated in DMEM supplemented with 160 nM insulin and subsequently incubated in DMEM medium containing 10% FBS, with replacement of medium with fresh medium every two days.

2.4. Western blot analysis

3T3-L1 cells were collected on the indicated days, washed twice with ice-cold phosphate-buffered saline (PBS), and lysed in RIPA buffer containing protease inhibitors (1 mM phenymethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) on ice for 1 h. Cell lysates were extracted, and proteins (30 μg) were separated on a 10% SDS-PAGE gel and analyzed by the subsequent western blotting as described [16]. Proteins were visualized using an ECL detection system (Thermo Scientific, Waltham, MA).

2.5. Oil Red O staining and quantification

Cells were washed twice with 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 in isopropanol:water) for 30 min at 25 °C and washed with water three times. Staining was visualized by bright-field microscopy, and Oil Red O extracted from cells in isopropanol solution containing 4% Nonidet P-40 was quantified by measuring absorbance at a wavelength of 520 nm.

2.6. Statistical analysis

Data are represented as mean (±SD) of at least three independent experiments. Difference between two groups was assessed by paired Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Overexpression of GFP-LC3 induces adipocyte differentiation of 3T3-L1 preadipocytes

Growth-arrested 3T3-L1 preadipocytes undergo adipogenesis by re-entering the cell cycle and undergoing subsequent mitotic clonal expansion in the presence of MDI. During the earlier stages of this process, many transcription factors, including C/EBPs and