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Original article

Rock signaling control PPARγ expression and actin polymerization during adipogenesis

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ABSTRACT

Aim: Adipogenesis is characterized by a strong interdependence between cell shape, cytoskeletal organization, and the onset of adipogenic gene expression. Here we investigated the role of the RhoA/ROCK pathway in adipogenesis. *Result:* High RhoA activity in the cell line C3H10T1/2 were generated (Named RhoA14V cells). Treatment of RhoA14V cells with Shield 1 following their differentiation into adipocytes resulted in the appearance of thick cortical actin filaments, and increased mRNA expression levels of RhoA, ROCK, p-MYPT1 and p-MLC, while PPARγ mRNA decreased. This resulted in decreased triglyceride synthesis and reduced expression of the adipogenic transcription factor PPARγ. These molecular changes were accompanied by reorganization of the actin cytoskeleton, during which ROCK signaling suppressed actin polymerization. *Conclusion:* ROCK signaling suppresses adipogenesis by controlling PPARγ expression and actin organization in adipocytes.

1. Introduction

Adipose tissue is a reservoir of fat that functions as the main form of energy storage (Ruan et al., 2014). In the past decade, adipose tissue and adipocytes have become a global focus of research (Kavalkova et al., 2013) Too little body fat caused by deficient fat uptake can lead to severe metabolic disorders, such as hypertriglyceridemia and early onset type II diabetes mellitus. However, fat accumulation causes obesity, which increases the risk of cardiovascular diseases, type II diabetes and metabolic syndrome (Fox, 2008).

Rho is a member of the Ras sub-family of the small GTPase super-family. Since the first report by Pascal Madaule in 1985, over 20 Rho family proteins have been discovered (Ridley, 2001). Rho family proteins are 20–25 kDa GTP-bound proteins with GTPase activity. These proteins are bound to GTP in the active state, and to GDP in the inactive state. Rho proteins regulate downstream signaling molecules by switching between these two state (Wennerberg and Der, 2004). By regulating the Rho kinase, ROCK, and its downstream proteins, MYPT and MLC, Rho regulates depolarization of the actin skeleton, which modulates cellular polarity and morphology, as well as adipogenesis. Rho proteins can be categorized into RhoA, Racl, Cdc24 and non-GTPase sub-families according to the degree of homology and function. Activation of RhoA is required for actin-based cytoskeletal contraction. G14V or Q63L mutations cause Rho to persist in the activated GTP-bound state due to impaired hydrolysis (Mack et al., 2001), thus enhancing the function of the mutant form. In this study, we investigated the role of Rho and RhoA/ROCK signaling in adipogenesis by overexpressing RhoA14V and inducing adipogenic differentiation in stable expression clones. Our results may provide a theoretical basis for modulation of body fat deposition and reveal a potential therapeutic target for obesity-related diseases.

2. Material and methods

2.1. Cell strains and cell culture

C3H10T1/2 was obtained from the Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China). The C3H10T1/2 cells were maintained in DMEM medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and cultured under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The Plat-E Retrovirus Packaging cell line was obtained from the Cell Biolabs. Plat-E cells were maintained in DMEM supplemented with 10%

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FBS) and containing 1 μg/mL puromycin, 10 μg/mL blasticidin, penicillin and streptomycin and cultured under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. C3H10T1/2 cells were grown to confluence and induced to differentiate into adipocytes by exposure to a cocktail containing DMEM supplemented with 10% FBS, 0.5 mM IBMX (Sigma, USA), 0.1 mM dexamethasone (Sigma), and 0.2 mg/L insulin and 0.5 mM rosiglitazone (Sigma) for 48 h. Subsequently, the cells were switched to differentiation medium (DMEM, 10% FBS, 0.1 mM dexamethasone (Sigma), and 0.2 mg/L insulin for 48 h. Cells were then cultured 4 or 8 days in DMEM with 10% FBS.

2.2. Oil-Red O staining and lipid quantification

At 4 or 8 days after differentiation treatment, removed the cells and treated with 4% paraformaldehyde for 35 min at RT. Cells were washed three times in phosphate buffered saline (PBS) and incubated with oil-red O working solution (0.5% oil-red O in isopropanol: deionized water mixed at 3:2 immediately before use) for 40 min. The cells were then washed with PBS and lipid droplets stained with oil-red O were extracted in 200 μL of absolute isopropyl alcohol for quantification by measuring the absorbance at 490 nm. Triglyceride levels were measured using a triglyceride (TG) assay kit according to the manufacturer's instructions. The TG assays were repeated in triplicate.

2.3. PCR method

Total RNA from C3H10T1/2 and RhoA14V cells was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Reverse transcription-PCR (RT-PCR) was performed using Fermentas K1622 RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). RT-qPCR was performed with a SYBR PrimeScript™ RT-PCR kit (TaKaRa) using the ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The sequence specific primers are showed in Table 1. The relative gene expression of each sample was shown.

2.4. Western blot

C3H10T1/2 cells were cultured on 60-mm plates for different periods of time, suspended and lysed in 200 μL lysis buffer containing a complete protease inhibitor cocktail and PhosStop (Roche, USA) for 30 min on ice. The lysates were centrifugated at 12,000 rpm for 5 min at 4 °C. Protein concentrations were quantified by the BCA protein assay kit (Beyotime, Shanghai, China), boiled 5 min mixed with a sample loading buffer before separation by SDS-PAGE. The proteins were then electro- transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA), blocked by incubation with 5% non-fat milk for 1 h and incubated with the primary antibodies for the detection of RhoA (67B9), ROCK2 (D1B1), MYPT1 (2364) (diluted 1:1,000; Cell Signaling Technology, Danvers, MA, USA); MLC (ab92721) P-MLC (ab2480) (diluted 1:5,000; Abcam); and GAPDH (diluted 1:500; Goodhere Biotechnology, Hangzhou, China) overnight at 4 °C. After washing, the membranes were then incubated with specific horseradish peroxidase-conjugated secondary antibodies and visualized using an electrochemiluminescence reagent.

Table 1
Primer sequences used in RT-**q**PCR.

Genes	Primers
PPARγ	S:5′-TGTCTCACAATGCCATCAGGTT-3′ A:5′-TGGTGATTTGTCCGTTGTCTTTC-3′
C/EBPα	S:5′-CAGAGGGACTGGAGTTATGACAAG-3′ A:5′-CCCAGCCGTTAGTGAAGAGTC-3′
RhoA	S:5′-GGATGGGAAGCAGGTAGAGTTG-3′ A:5′-CGGCTCCTGCTTCATTTTGG-3′
β-actin	S: 5′-CAGCCTTCCTTCTTGGGTATGG-3′ A:5′-TGTGTTGGCATAGAGGTCTTTACG-3′

2.5. Statistical analysis

All data are presented as the means ± SD and were analyzed by the SPSS 22.0 software package. Differences between groups were examined for statistical significance using unpaired Student's *t*-tests. Multiple comparisons were analyzed by one-way ANOVA. $P < .05$ was considered to indicate statistical significance.

3. Results and discussion

3.1. Lipid quantification by oil-red O staining

RhoA14V and control C3H10T1/2 cells following the induction of adipogenic differentiation stained by oil red O. The number of red-stained lipid droplets increased with time during the process of adipogenic differentiation in both RhoA14V and C3H10T1/2 cells. Furthermore, the cell morphology changed from the original fibroblast-like appearance to an irregular shape and finally, to oval in the late stage of differentiation. Notably, there were fewer lipid droplets with smaller volumes in the RhoA14V cells than in the control C3H10T1/2 cells. At 4 d, numerous lipid droplets were visible in the cytoplasm of C3H10T1/2 cells, while only tiny lipid droplets were observed in some of the RhoA14V cells. At 8 d, the lipid droplets in the control C3H10T1/2 cells became were larger and more numerous, and they formed a circle surrounding the nucleus (Fig. 1).

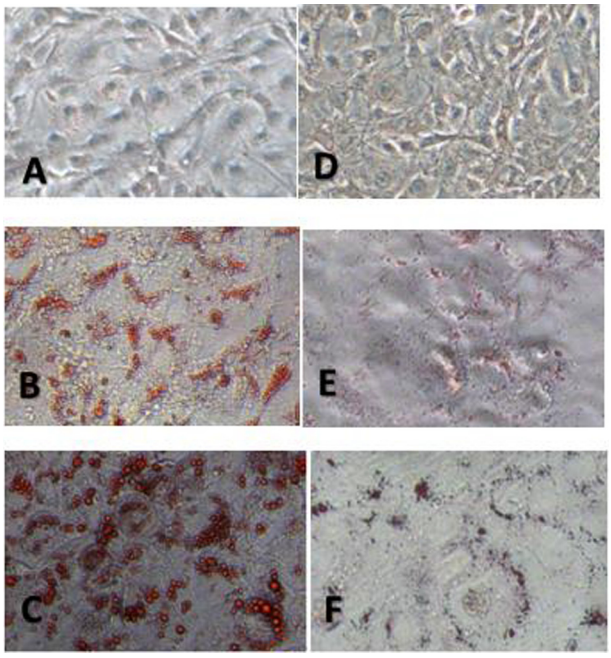

Fig. 1. Oil-red O staining of C3H10T1/2 cells at different time-points after the induction of adipogenic differentiation (oil-red O staining, 200×). A: Confluent C3H10T1/2 cells on day 0 (200×); B: C3H10T1/2 cells stained with oil-red O on day 4 (200×); C: C3H10T1/2 cells stained with oil-red O at 8 d (200×); D: Confluent RhoA14V cells at 0 d (200×); E: RhoA14V cells stained with oil-red O at 4 d (200×); F: RhoA14V cells stained with oil-red O at 8 d (200×).