Biochemical and Biophysical Research Communications 434 (2013) 455–459

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Retinoic acid inhibits adipogenesis via activation of Wnt signaling pathway in 3T3-L1 preadipocytes

Dong Min Kim [a], Hye-Ryung Choi [b], Anna Park [b], Sang-Mo Shin [a], Kwang-Hee Bae [b], Sang Chul Lee [b], Il-Chul Kim [c,*], Won Kon Kim [b,*]

[a] Department of Medical System Engineering, Gwangju Institute of Science and Technology, 123 Cheomdan-gwagiro, Buk-gu, Gwangju 500-712, Republic of Korea
[b] Medical Proteomics Research Center, KRIBB, Daejeon 305-806, Republic of Korea
[c] Department of Biological Sciences, Chonnam National University, Gwangju 500-757, Republic of Korea

ARTICLE INFO

Article history:
Received 11 March 2013
Available online 10 April 2013

Keywords:
Adipogenesis
Retinoic acid
Wnt/β-catenin
Signaling pathways

ABSTRACT

Although retinoic acid (RA) is well known to inhibit the differentiation of 3T3-L1 cells into adipocytes both *in vivo* and *in vitro*, its molecular mechanism is not fully understood. In this report, we investigate the inhibitory mechanism of adipocyte differentiation by RA in 3T3-L1 cells. Because both RA and Wnt are known to inhibit adipogenesis at a common step involving the inhibition of PPAR-γ expression, we focused on the crosstalk between these two signaling pathways. We found that RA treatment resulted in a dramatic inhibition of adipogenesis, especially at an early phase of differentiation, and led to increased β-catenin protein expression. Moreover, RA enhances the transcriptional activity of β-catenin as well as Wnt gene expression during adipogenesis. Taken together, the present study demonstrated that Wnt/β-catenin signaling may be associated with the RA-induced suppression of adipogenesis and may cooperatively inhibit adipocyte differentiation.

1. Introduction

Obesity is increasingly considered as a prevalent health problem in industrialized countries because obesity is closely associated with the prevalence of diabetes and cardiovascular disease. Obesity is caused by the increase of adipose tissue that results from lipid accumulation in preexisting adipocytes and an increased number of adipocytes through the proliferation and differentiation of preadipocytes [1–3].

The differentiation of preadipocytes into mature adipocytes has been widely studied with an *in vitro* model using the 3T3-L1 preadipocyte cell line, which was isolated from disaggregated mouse embryos based on the propensity of these cells to differentiate into adipocytes in culture [4–6]. The program of adipogenesis begins upon the treatment of confluent 3T3-L1 preadipocytes with an adipogenic medium containing methylisobutylxanthine, dexamethasone, insulin (MDI), and fetal calf serum [4,7,8]. This treatment initiates early events in adipogenesis, including the up-regulation of CCAAT/enhancer binding protein-β (C/EBP-β) and C/EBP-δ [6,9,10]. C/EBP-β and C/EBP-δ mediate the transcriptional activation of two master regulators of adipocyte differentiation, peroxisome proliferator-activated receptor-γ (PPAR-γ) and C/EBP-α. These master factors cooperate to promote the expression of adipogenic genes, leading to the terminal differentiation of adipocytes. On the other hand, the inducers of adipocyte differentiation promote the down-regulation of inhibitory factors such as several members of the GATA family and Wnt family [11–14]. The Wnt signaling pathway serves to inhibit the differentiation of preadipocytes. For instance, sustained overexpression of Wnt-1 or Wnt-10b blocks adipogenesis in the 3T3-L1 preadipocyte through the activation of β-catenin [11,12]. In addition, the suppression of adipogenesis by Wnt signaling was found during any of the first 3 days of differentiation [11], suggesting that Wnt signaling plays a key role in the early stages of adipocyte differentiation.

Retinoic acid (RA) has long been known as another inhibitor of adipocyte differentiation [15–19]. RA exerts its inhibitory roles during the differentiation of 3T3-L1 cells by blocking the C/EBP-β-mediated transcription and induction of PPAR-γ [20]. Also, RA inhibits the adipogenesis of 3T3-L1 cells via RA receptor signaling [19,21] and Smad3 transcriptional activation [22]. Recently, Lee et al. [23] reported that RA blocked the adipogenic differentiation of BMP4-induced C3H10T1/2 cells via the down-regulation of Smad/p38MAPK signaling. Other research demonstrated that Erk signaling also regulates the RA-induced adipogenic differentiation of ES cells [15]. Thus, various adipogenic signaling pathways are involved in the RA-dependent regulation of adipogenesis.

* Corresponding authors. Fax: +82 42 879 8596.
E-mail addresses: ickim@chonnam.ac.kr (I.-C. Kim), wkkim@kribb.re.kr (W.K. Kim).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.03.095

Given that both RA and Wnt are known to inhibit adipogenesis at a common step involving the inhibition of PPAR-γ expression, we focused on the crosstalk between these two signaling pathways. In so doing, we demonstrated that RA blocks adipogenesis by stabilizing β-catenin signaling and that β-catenin is activated through the increased expression of the members of the Wnt gene family.

2. Materials and methods

2.1. Cell cultures and adipogenic differentiation

3T3-L1 cells were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM of D-glucose supplemented with 10% (v/v) bovine calf serum and a 1% antibiotic–antimycotic solution (Gibco-Invitrogen, USA) at 37 °C in a humidified atmosphere with 5% CO_2.

For adipogenic differentiation, 3T3-L1 preadipocytes were induced to differentiate into mature adipocytes as described previously [24,25]. Briefly, cells were placed in differentiation medium consisting of DMEM, 10% fetal bovine serum (FBS) and MDI [a differentiation cocktail of 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone and 10 μg/ml insulin (all from Sigma–Aldrich, USA)]. After 48 h, the medium was switched to a maintenance medium composed of DMEM, 10% FBS and 10 μg/ml insulin. The medium was replenished every other day. RA and troglitazone (Sigma–Aldrich, USA) were added at concentrations of 5 and 10 μM, respectively.

2.2. Oil-Red-O staining

Oil-Red-O staining was performed following a procedure described previously [24–26]. The cells were washed with phosphate-buffered saline (PBS), fixed in 10% formalin, and stained with 0.3% of filtered Oil-Red-O solution in 60% isopropanol. The cells were then photographed using phase-contrast microscopy.

2.3. Immunoblot analysis

Cultured cells were rinsed with ice-cold phosphate buffered saline (PBS) and harvested in ice-cold RIPA or NP-40 lysis buffer containing a protease-inhibitor (Roche, Switzerland). Protein concentrations were measured with the BSA assay (Bio-Rad, USA). SDS–PAGE and Western blots were performed using standard protocols. β-Catenin and aP-2 (FABP4) antibodies were purchased from Cell Signaling. Anti-β-actin and α-tubulin were obtained from Sigma. Secondary antibodies were purchased from Abcam, and the membranes were visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, USA).

2.4. RT-PCR and real-time PCR

Total RNA was extracted from 3T3-L1 preadipocytes using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. After quantification, mRNA was assayed in equivalent amounts of total RNA by RT-PCR. RT reactions were performed from 2 μg total RNA using oligo (dT) primers in a total volume of 20 μl, according to the manufacturer's instructions. The primer sequences were as follow: mouse Wnt-1 (forward, 5′-GGGTTTCTACTACGTTGCTA-3′, and reverse, 5′-CTCACGCTGTGCAGGATCCC-3′); mouse Wnt-4 (forward, 5′-GAGCAATTGGCTGTACCTGG-3′, reverse 5′-AGTGTGGAA TTCCAGCG-3′). The PCR was performed with the Maxime PCR premix kit (Intron, Korea), and PCR products were analyzed by 1% (w/v) agarose gel electrophoresis and revealed with ethidium bromide.

For real-time PCR, two times real-time PCR smart mix (Solgent, Korea) was applied to detect the aP-2 and PPAR-γ expression levels using the CFX96™ Real-Time System (Bio-Rad, USA). Primers were used for mouse aP-2 (forward, 5′-ACACCGAGATTTCCTTCAA-ACTG-3′, reverse, 5′-CCATCTAGGGTTATGATGCTCTTC-3′) and PPAR-γ (forward, 5′-CAAGAATACCAAAGTGCGATCAA-3′, reverse, 5′-GAGCTGGGTCTTTTCAGAATAATA-3′).

2.5. Luciferase reporter assay

The cells were seeded in six-well plates in triplicate for 24 h before transfection. 200 ng of TOP-flash or FOP-flash was transfected into each well using Lipofectamin (Invitrogen). Three hours later, 5 μM RA was introduced into each well. The cells were washed with PBS and lysed with 500 μM of passive lysis buffer (DLR™ Assay System, Promega). The average ratio from a triplicated sample of luciferase activity was calculated.

3. Results

3.1. RA inhibits lipid accumulation and stimulates β-catenin expression during the adipogenic differentiation of 3T3-L1 cells

To establish whether RA influences the adipogenesis of 3T3-L1 cells, we investigated the effect of RA on adipocyte differentiation. The 3T3-L1 preadipocytes initiated their differentiation to adipocytes upon treatment with MDI. After changing the medium, cultivation of these cells for another 8 days fully differentiated adipocytes. On the other hand, compared to control cells, cells treated with RA dramatically suppressed adipogenic differentiation (Fig. 1A). Consistently, the expression levels of the adipogenic markers of such as aP-2 and PPAR-γ were also reduced in mature adipocytes in response to the treatment with RA (Fig. 1B and C). For a more detailed examination of the RA effect, RA was added to the culture medium at different time points during adipogenic differentiation. As shown in Fig. 1D, the presence of RA at an early stage (0–2 days) was shown to inhibit lipid accumulation, whereas at the terminal stage (6–8 days) no change was noted as regards the subsequent lipid accumulation. Thus, anti-adipogenic effect of RA is an effective early step of adipogenesis.

Next we examined the effect of RA on β-catenin expression. The expression of β-catenin was down-regulated during adipogenesis, indicating consistency with previous observations that the reduction of β-catenin is essential for the differentiation of preadipocytes. On the other hand, the RA treatment resulted in an increase of β-catenin with a concomitant inhibition of adipogenesis, suggesting that a decrease of β-catenin and the inhibition of adipogenesis are not independent and parallel events caused by RA (Fig. 1B). Therefore, it is likely that the RA-induced accumulation of β-catenin contributes to the inhibition of adipogenesis.

3.2. RA stimulates the expression of Wnt/β-catenin signaling components

To determine how RA stimulates the accumulation of β-catenin in 3T3 cells, we initially investigated the effects of RA on the mRNA level of β-catenin during early adipogenesis, as RA affected 3T3-L1 adipogenesis at an early phase of differentiation. As shown in Fig. 2C, the mRNA expression of β-catenin did not change upon RA treatment. However, a Western blot analysis showed the accumulation of β-catenin protein (Fig. 2A), indicating that the increase of β-catenin by the RA treatment was mediated by β-catenin stabilization.