



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

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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.

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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.

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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% CO₂.

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 μ M dexamethasone and 10 μ g/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 μ g/ml insulin. The medium was replenished every other day. RA and troglitazone (Sigma–Aldrich, USA) were added at concentrations of 5 and 10 μ M, 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 μ g total RNA using oligo (dT) primers in a total volume of 20 μ l, according to the manufacturer's instructions. The primer sequences were as follow: mouse Wnt-1 (forward, 5'-GGGTTTCTACTACGTTGCTA-3', and reverse, 5'-CTCAGCTGTGCAGGATCCC-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'-ACACCAGATTTCTTCAA-ACTG-3', reverse, 5'-CCATCTAGGGTTATGATGCTCTTC-3') and PPAR- γ (forward, 5'-CAAGAATACCAAAGTGGCATCAA-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 μ M RA was introduced into each well. The cells were washed with PBS and lysed with 500 μ M 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.

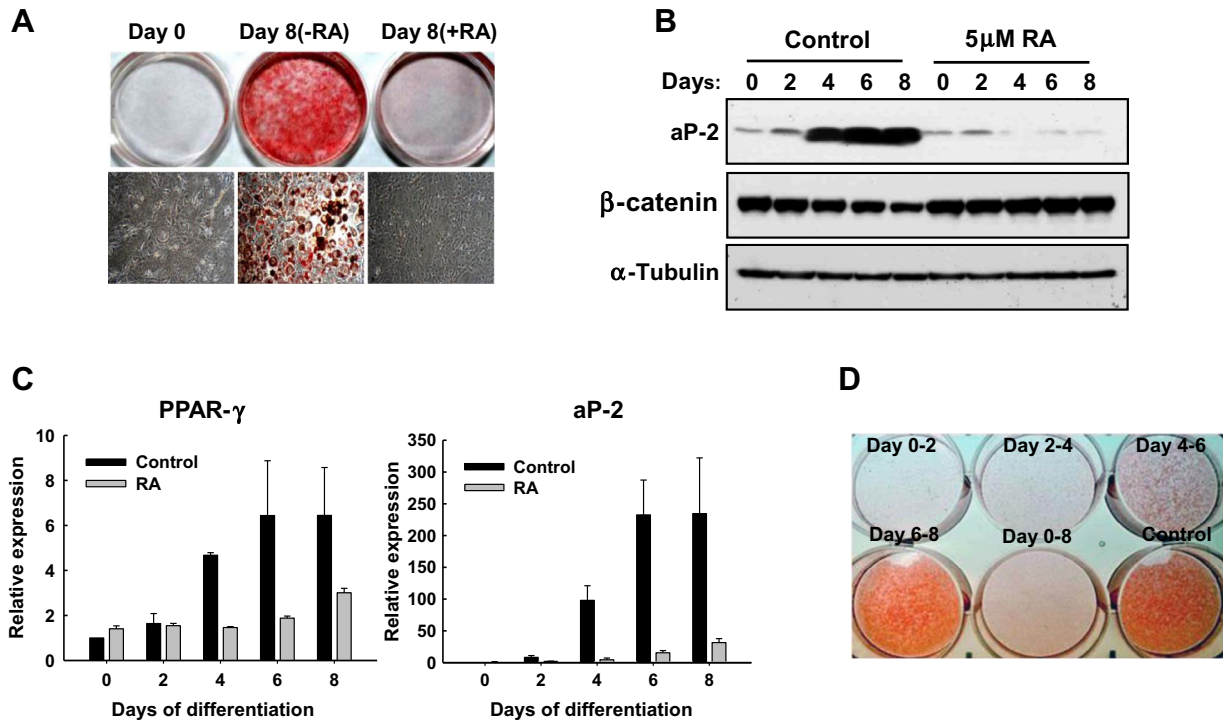


Fig. 1. RA inhibits adipogenesis and stimulates the accumulation of β -catenin in 3T3-L1 adipocytes: (A) cells were induced to differentiate into mature adipocytes with MDI for 8 days, followed by treatment with 5 μ M RA every other day. After 8 days, the cells were stained with Oil-Red-O to visualize the degree of lipid accumulation. (B) Protein levels of β -catenin and aP-2 during adipogenic differentiation of 3T3-L1 cells were examined by Western blot analysis. Whole-cell lysates were extracted on the indicated days of differentiation in the absence or presence of 5 μ M RA. α -Tubulin was used as a loading control. (C) Analysis of adipocyte-specific gene expression during adipogenesis of 3T3-L1 cells with or without RA. Total RNA were extracted on the indicated days for real-time PCR. (D) RA was added to the culture medium at different time points during adipocyte differentiation. After 8 days of differentiation, lipid accumulation was assessed by Oil-Red-O staining.

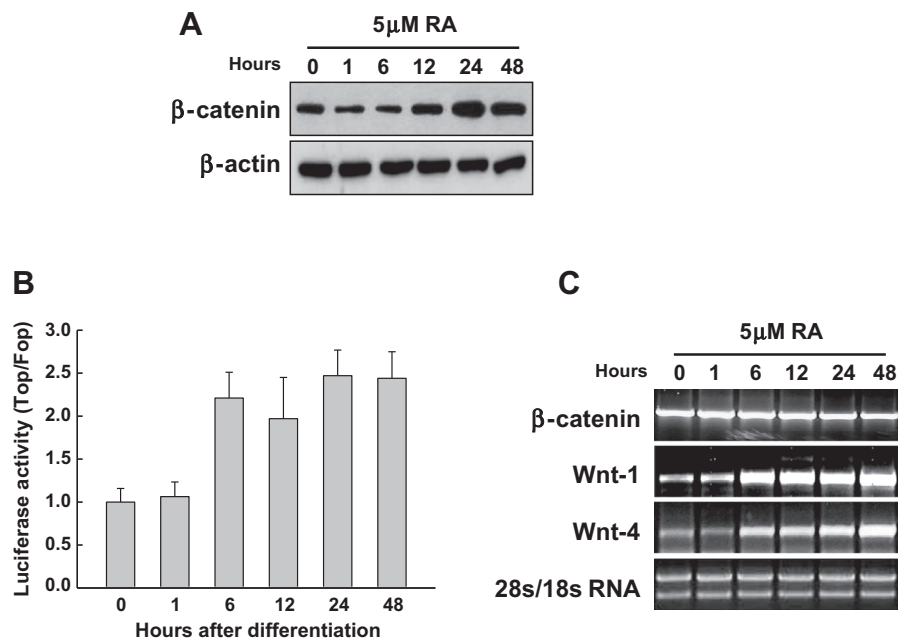


Fig. 2. Effects of RA on the expression of the Wnt/ β -catenin signaling components: (A) analysis of β -catenin protein expression after an RA treatment at an early phase of adipogenic differentiation. (B) Analysis of TOP reporter assay results upon an RA treatment of 3T3 cells. Sub-confluent 3T3-L1 cells were transfected with Top-flash or Fop-flash and *Renilla* luciferase reporters. Three hours later, 5 μ M RA was introduced into each well, and lysates were isolated at the indicated times. The luminescence of the lysates was determined and the average ratio from triplicated samples was calculated. (C) Analysis of the mRNA expression of β -catenin and Wnts genes in the presence of 5 μ M RA during the early phase of adipogenesis. 28s/18s RNA was used as a loading control.

To confirm that RA activates the transcription or enhances the transcriptional activity of β -catenin during adipogenesis, we performed a TOP reporter assay of the RA treatment in 3T3 cells.

The transcription of the Top reporter gene increased 6 h after the RA treatment in 3T3 preadipocyte cells (Fig. 2B), showing good agreement with the kinetics of β -catenin accumulation.

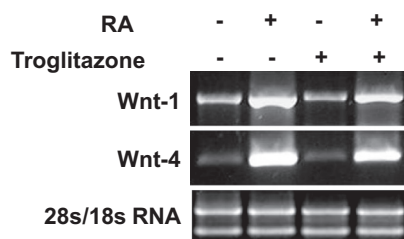


Fig. 3. Effect of a troglitazone treatment on Wnt gene expression in 3T3-L1 cells. Confluent 3T3-L1 preadipocytes were exposed to DEX and insulin for 48 h and then treated with or without 10 μ M troglitazone and/or 5 μ M RA for 24 h. Messenger RNA levels of Wnt-1 and Wnt-4 were examined by RT-PCR.

Next, we examined the effects of RA on the mRNA expression of Wnt/ β -catenin signaling components, including multiple Wnts, secreted frizzled-related peptides (sFRPs), TCF-4 and LEF-1. We exposed 3T3-L1 cells to 5 μ M RA, after which the RNAs were isolated from independent cultures treated with RA for 0, 1, 6, 12, 24, or 48 h. As noted by RT-PCR of the RNAs, most of the Wnt/ β -catenin signaling components did not show a change in the mRNA level upon RA treatment in 3T3-L1 cells (data not shown). However, the RA treatment resulted in a dramatic increase in the Wnt-1 and Wnt-4 mRNA levels, showing good correlation with the result of the Top reporter assay (Fig. 2C). Therefore, it appears that the RA-induced expression of Wnts contributes to the accumulation of β -catenin and consequently the inhibition of adipogenesis.

3.3. The expression of Wnt cannot be reversed by the exposure of cells to the PPAR- γ ligand troglitazone

On the basis of a report that a treatment with the PPAR- γ ligand troglitazone decreased β -catenin expression during adipogenesis and reduced the β -catenin level is recovered by a proteasome-dependent mechanism [27], we investigated whether the activation of PPAR- γ could reverse the RA-induced up-regulation of Wnt-1 and Wnt-4 expression, as Wnt also inhibits the proteasome-dependent degradation of β -catenin. As shown in Fig. 3, however, the expression of Wnt could not be reversed by exposure of the cells to the troglitazone, suggesting that the increase in the Wnt genes by RA is an event that occurs upstream of PPAR- γ signaling.

4. Discussion

Adipocyte differentiation is controlled by positive and negative stimuli such as various chemicals, enzymes, cytokines and

transcription factors [6,10,28]. RA is a metabolite of vitamin A that plays an important role in cell growth and development [29,30]. In adipogenesis, the role of RA is widely recognized [15–19]. In this study, we investigated the inhibition mechanism of adipocyte differentiation by RA. Wnt/ β -catenin signaling is also known to inhibit adipogenesis at the step associated with the inhibition of PPAR- γ expression in preadipocytes. Moreover, β -catenin expression is increased by RA treatment in mouse epiphyseal chondrocytes [31]. Therefore, we proposed that RA affects β -catenin signaling while blocking the differentiation of 3T3-L1 preadipocytes. As expected, following the RA treatment, adipocyte differentiation was markedly decreased and led to increased β -catenin expression in our experimental conditions. These results indicate that the anti-adipogenic function of RA is induced through the regulation of β -catenin expression. In addition, the presence of RA after 0–2 days of differentiation lead to a dramatic inhibition of adipogenesis, indicating that RA regulates the early stage of adipogenic differentiation. On the other hand, RA did not change β -catenin expression, suggesting that RA affects adipogenesis via β -catenin stabilization.

The activation of β -catenin can be regulated in several ways, including changes in the expression and/or phosphorylation of the Wnt signaling components. Interestingly, we found that the RA treatment resulted in a dramatic increase in the Wnt-1 and Wnt-4 mRNA levels, showing a good correlation with the increase in the transcriptional activity of β -catenin (Fig. 2). These data suggest that the RA-induced expression of the Wnts gene contributes to the inhibition of adipogenesis. However, it is not clear whether Wnt/ β -catenin signaling during the RA-induced inhibition of adipogenesis is a key intracellular signaling pathway, as RA-dependent regulation of adipogenic differentiation has other pathways, such as the Erk and Smad/p38 pathways [15,23]. Thus, extensive studies are necessary to clarify the detailed mechanism of the RA-induced inhibition of adipogenesis. The good correlation between the accumulation of β -catenin by an RA treatment and the induction of Wnt-1 and Wnt-4 transcription, however, suggests that Wnt/ β -catenin signaling is at least one of the major pathways through which RA inhibits the adipogenesis of 3T3-L1 cells.

PPAR- γ is considered as a principal component in the adipogenesis of 3T3-L1. Previous studies demonstrated that the activation of PPAR- γ led to a decrease of β -catenin expression during adipogenesis; this reduction of β -catenin is involved via a proteasome-dependent degradation mechanism [27]. In general, Wnt exerts its functional role during the accumulation of β -catenin by inhibiting the proteasome-dependent degradation of β -catenin. Therefore, we speculate that the activation of PPAR- γ could reverse Wnt-1 and Wnt-4 expression as induced by RA. Contrary to our expectations, Wnt could not be reversed by the exposure of cells to the PPAR- γ ligand troglitazone, suggesting that the induction

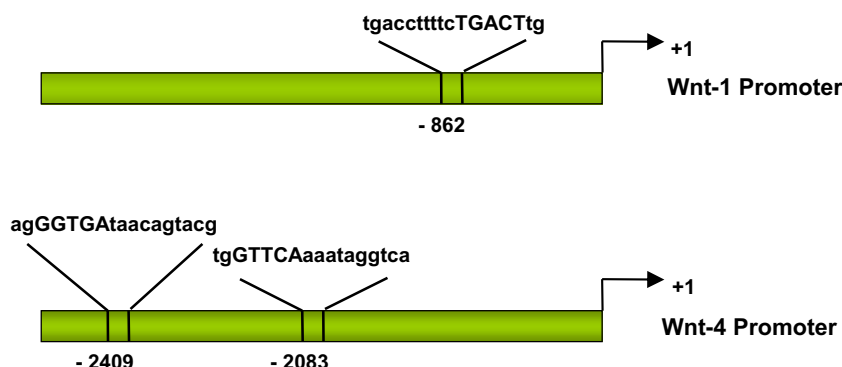


Fig. 4. Schematic representation of the RAR/RXR binding sequence on the promoter region of Wnt-1 and Wnt-4 genes.

of Wnt by RA is independent of the PPAR- γ activity during the differentiation process of the adipocyte.

The effects of RA are mediated by specific nuclear receptors of the RA receptor family (RARs) and retinoid X receptor family (RXRs) [32,33]. In response to the binding of RA, RAR/RXR heterodimers recognize and bind to DNA response elements in the regulatory region of target genes (RAREs). Ligand binding recruits coactivators and general transcription factors to activate transcription of the target genes [32,34]. As such, we searched for RAR and RXR binding sites throughout the –3 kb region on promoters of the Wnt-1 and the Wnt-4 genes. As a result, we found that both the Wnt-1 and Wnt-4 genes have a RAR/RXR binding site on their promoters (Fig. 4). Furthermore, the binding sites on the Wnt-1 and Wnt-4 gene are conserved in other mammalian species, such as the mouse and rat (data not shown). These results strongly suggest that RAR/RXR heterodimers regulate the expression of Wnt-1 and Wnt-4 genes directly by binding to their promoter which responds to a RA treatment.

In conclusion, we demonstrated that RA blocks adipogenesis by stabilizing β -catenin signaling and the transcriptional activation of β -catenin. We also correlated this with the increased expression of the members of the Wnt gene family. Our data suggest that Wnt/ β -catenin and RA signaling pathways are connected and cooperatively inhibit adipocyte differentiation.

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