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Molecular mechanism of 9-*cis*-retinoic acid inhibition of adipogenesis in 3T3-L1 cells

Chiaki Sagara [a], Katsuhiko Takahashi [a], Hiroyuki Kagechika [b], Noriko Takahashi [a,*]

[a] Laboratory of Physiological Chemistry, Institute of Medicinal Chemistry, Hoshi University, Shinagawa, Tokyo 142-8501, Japan
[b] School of Biomedical Science, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda, Tokyo 101-0062, Japan

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ABSTRACT

Retinoic acid (RA) signaling is mediated by specific nuclear hormone receptors. Here we examined the effects of 9-*cis*-RA on adipogenesis in mouse preadipocyte 3T3-L1 cells. 9-*cis*-RA inhibits the lipid accumulation of adipogenetically induced 3T3-L1 cells. The complex of retinoid X receptor α (RXRα) with peroxisome proliferator-activated receptor γ (PPARγ) is a major transcription factor in the process of adipogenesis, and the levels of these molecules were decreased by 9-*cis*-RA treatment. A RXR pan-antagonist suppressed 9-*cis*-RA's inhibitory effects on adipogenesis, but not on the intracellular levels of both RXRα and PPARγ. These results suggest that 9-*cis*-RA could inhibit adipogenesis by activating RXR, and decrease both RXR and PPARγs levels in a RXR activation-independent manner.

1. Introduction

Obesity is the leading risk for the development of type 2 diabetes, hypertension and cardiovascular disease. Understanding the molecular mechanisms of adipogenesis has implications on obesity and insulin resistance [1]. The mouse preadipocyte cell line, 3T3-L1, exhibits differentiation processes of mature adipocytes following activation of the adipogenetic signaling pathway. The temporal pattern of expression of the various important adipogenic factors is dependent on transcription factors [2]. PPARγ is an obligatory key regulator, which functions as a transcription factor for many adipocyte-specific genes involved in lipid synthesis, insulin signaling and adipocytekine production for terminal differentiation [3,4]. Of the two isoforms, PPARγ1 and PPARγ2, PPARγ2 is the more highly adipocyte-specific type.

PPARγ forms a heterodimer with RXR. Since several reports have indicated that agonistic PPARγ ligands induce 3T3-L1 cells to differenciate into mature adipocytes, it has been suggested that RXR agonists could also be inducers of adipogenesis [5]. Nadzan et al. have shown that synthetic RXR-specific ligands are potent inducers of adipogenesis in 3T3-L1 cells [6]. On the other hand, Fushiki's cohort study examining the effects of vitamin A and its precursors, carotenoids and retinoids, on terminal differentiation of preadipocytes, showed that these compounds exhibit suppressive effects on adipocyte differentiation through RARγ up-regulation and the suppression of PPARγ [7].

Vitamin A is an important nutrient that supports several vital biological functions, including vision, embryonic development, reproduction, and immune function [8–10]. The bioactive form of vitamin A, all-*trans*-RA (ATRA), is a nutrient derivative with many remarkable effects on adipocyte biology affecting preadipocyte survival, and adipogenesis of preadipocyte clonal cell lines [11,12]. ATRA treatment reduces body weight and adiposity [13,14]. Retinoic acid (RA) regulates transcription mainly through binding and activation of two types of nuclear receptors, RAR and RXR [15,16]. ATRA is a specific RAR ligand that suppresses adipogenesis, in part through RAR activation.

The retinoid, 9-*cis*-retinoic acid (9-*cis*-RA), regulates the expression of retinoid responsive genes, serving as ligands for both RARs and RXRs. 9-*cis*-RA has been demonstrated to exhibit high-affinity RXR binding *in vitro* [17]. RXR forms tetramers in the absence of its ligand, and 9-*cis*-RA makes the RXR tetramer dissociate into RXR homodimers. RXR is an obligate heterodimeric nuclear receptor partner that can associate with other nuclear receptors, including PPARs, RARs, and VDR [18]. These RXR heterodimers take part in all stages of adipogenesis.

While some reports have previously indicated inhibitory effects of ATRA on adipogenesis and lipid accumulation, the influence of

Abbreviations: RA, retinoic acid; PBS, phosphate-buffered saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2); EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; VDR, vitamin D receptor; RAR, retinoic acid receptor; DIM, dexamethasone, insulin, 3-isobutyl-1-methylxanthine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

* Corresponding author. Address: Laboratory of Physiological Chemistry, Institute of Medicinal Chemistry, Hoshi University, 2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan. Fax: +81 3 5498 5950.
E-mail address: t-noriko@hoshi.ac.jp (N. Takahashi).

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9-*cis*-RA on adipogenesis has yet to be determined. In the current study, we examine the effects of 9-*cis*-RA on adipogenesis and lipid accumulation in a 3T3-L1 differentiation model. We had expected that 9-*cis*-RA would increase adipogenesis similar to synthetic RXR-specific ligands. However, 9-*cis*-RA unexpectedly inhibited adipogenesis in 3T3-L1 cells, and suppressed increases in intracellular levels of both RXRα and PPARγ. These results suggest that 9-*cis*-RA could inhibit adipogenesis by activating RXR, and decreasing both RXRα and PPARγs in a RXR activation-independent manner.

2. Materials and methods

2.1. Cell culture

Murine preadipocyte, 3T3-L1 cells were obtained from Dr. Fumio Fukai (Department of Pharmaceutical Sciences, Tokyo University of Science), and were routinely cultured in growth medium consisting of DMEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycine (Invitrogen) in 5% CO_2 incubator at 37 °C.

2.2. Adipocyte differentiation and retinoid treatment

Routinely cultured 3T3-L1 cells were differentiated according to an established protocol [19]. Briefly (Fig. 1A), the cells were cultured to full confluence. At 2 days post confluence, when we prepared both total RNA and whole cells lysates of "No treatment (NT)", the cells were stimulated with induction medium consisting of growth medium containing DIM (1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 1 μg/ml insulin). After 20 h, when we prepared the samples of "DIM" (day 0), cells were switched to insulin medium consisting of growth medium with 1 μg/ml insulin. DIM-stimulated cells were maintained in insulin medium with additional medium-changing at the third (3d) and the fifth day (5d) after the initial switching of medium. The cells normally differentiated into mature adipocytes by 7 days-culture with insulin medium (7d).

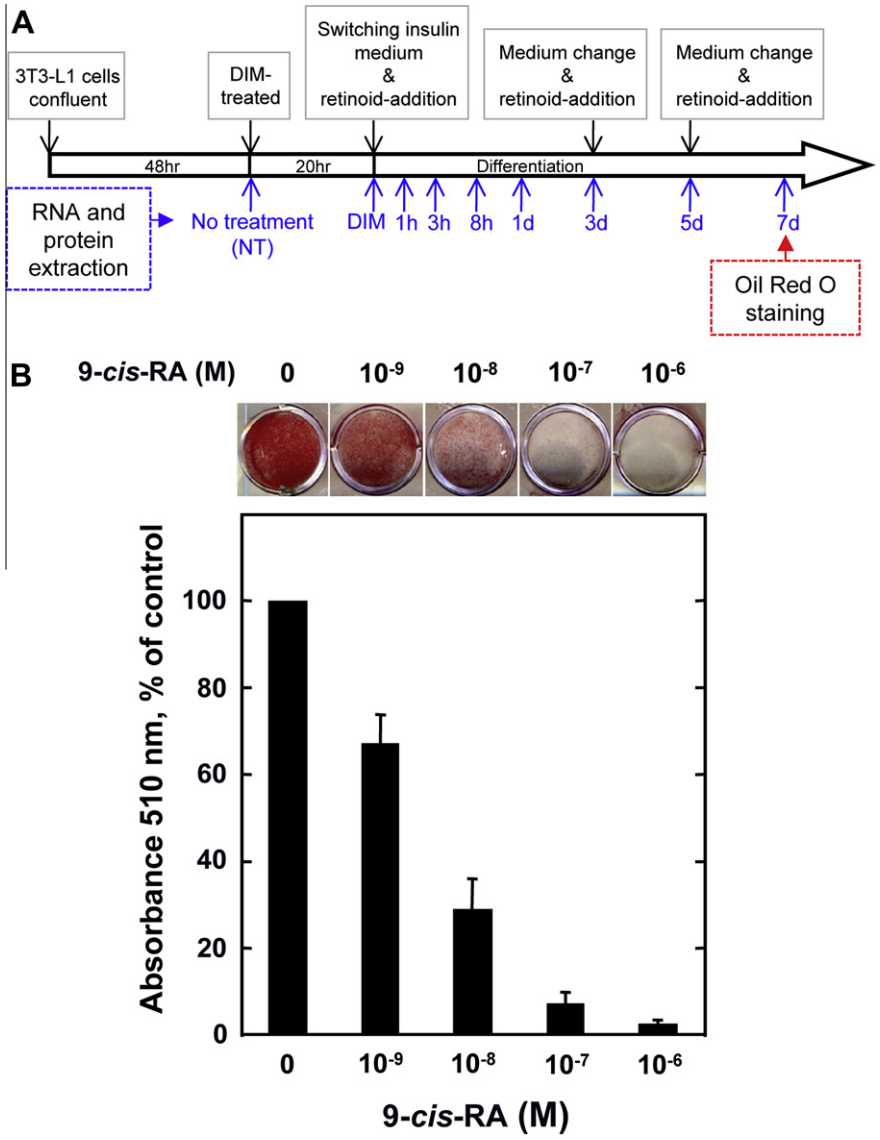

Fig. 1. Experimental schedule and inhibitory effects of 9-*cis*-RA on adipogenesis of 3T3-L1 cells. (A) 3T3-L1 preadipocytes were grown until near confluence, and then adipogenesis was induced using DIM. After 20 h (initial differentiation), 9-*cis*-RA was added and total RNA or proteins were extracted at 1 h, 3 h, 8 h, 1 day, 3 days, 5 days, and 7 days. (B) DIM-stimulated 3T3-L1 cells were treated with 9-*cis*-RA for 7 days. The accumulated lipid bodies were stained with Oil Red O. The photos show Oil Red O stained wells, and the corresponding graphs show sub-quantitative estimations of Oil Red O levels expressed as lipid body amounts. Each bar represents the mean ± SD.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In order to examine the effects of retinoids on adipogenesis, each retinoid or DMSO (vehicle) was added to the insulin medium. In brief, 100 nM 9-*cis*-RA (Sigma Chemical Co., St. Louis, MO, USA) or 100 nM of the RXR agonist HX630 [20], were added on the day of switching to insulin-containing medium (day 0). DIM-stimulated cells were treated with retinoids for 7 days with additional changing of the medium with retinoids at 3d and 5d. When we examine the effects of retinoid receptor antagonists on 9-*cis*-RA's effect on adipogenesis, we used LE540 as the RAR antagonist [20] and PA452 as the RXR antagonist [21]. The final concentrations of these antagonists were either 1 μM or 10 μM in the insulin medium containing 100 nM 9-*cis*-RA.

2.3. Oil Red O staining

3T3-L1 cells were fixed with 10% formalin for 10 min at room temperature. Cells were then washed with PBS and 60% isopropyl alcohol, followed by treatment with Oil Red O (2.1 mg/ml, Wako Pure Chemical Industries, Ltd.) for 30 min at room temperature. Samples were then washed twice with 60% isopropyl alcohol and once with PBS. To evaluate the accumulated lipid bodies, the stained plates were treated with 100% isopropyl alcohol to extract Oil Red O, and the solution was then measured for absorbance at 510 nm.

2.4. Immunoblotting analysis

Whole lysates of cells were prepared with the NP40 lysis buffer [22]. The protein concentrations of the whole cell lysates were determined by the Bicinchoninic Acid (BCA) Protein Assay (Piece, Rockford, IL, USA). Whole lysates were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were probed with either polyclonal antibodies against PPARγ (Cell Signaling, Danvers, MA, USA), monoclonal antibodies against RXRα (Perseus Proteomics, Tokyo, Japan) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were visualized by ECL-plus (Invitrogen).

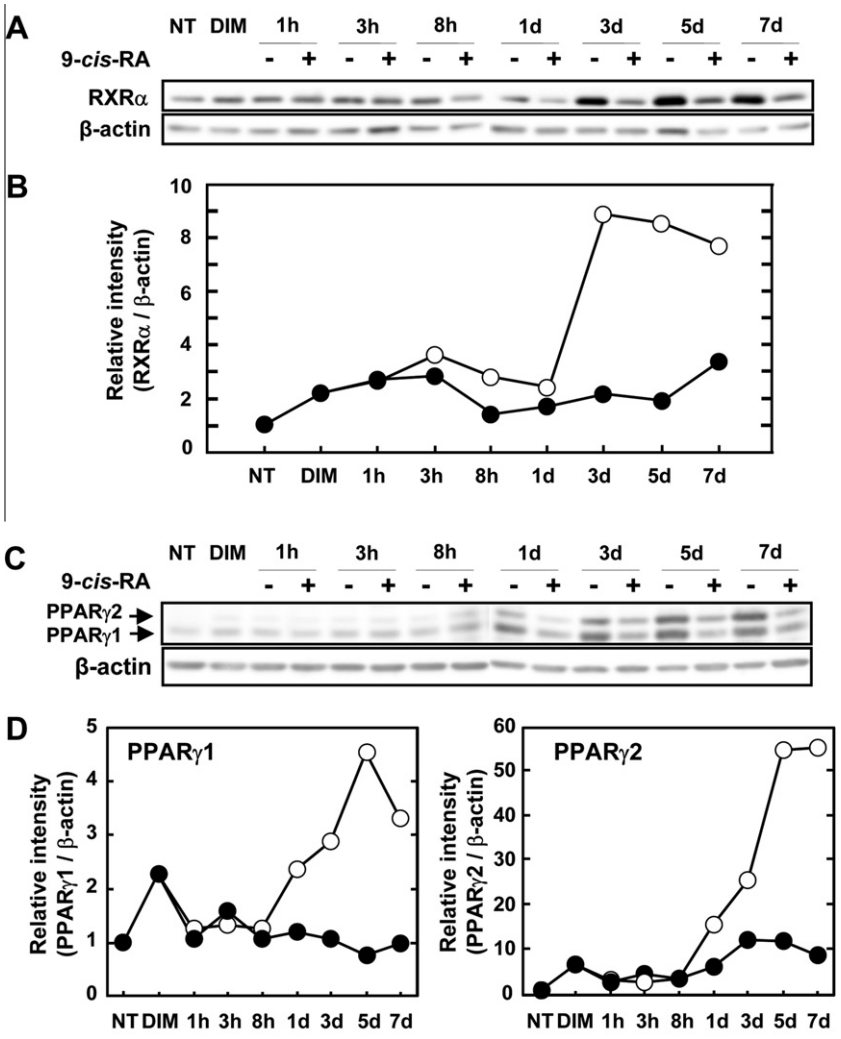

Fig. 2. 9-*cis*-RA-suppression of the increase in intracellular RXRα and PPARγs levels during adipogenesis. Whole lysates of 3T3-L1 cells were prepared prior to DIM-stimulation (NT) and at 20 h following DIM-stimulation (DIM), and at the indicated periods after addition of 100 nM 9-*cis*-RA (+) or 0.1% DMSO (−). (A) The intracellular levels of RXRα were detected with immunoblotting as described under Section 2. Upper photo shows RXRα and lower photo shows β-actin. (B) Relative levels of RXRα in each period. The relative RXRα levels were normalized with β-actin. 0.1% DMSO (○); 100 nM 9-*cis*-RA (●). (C) The intracellular levels of PPARγs were detected with immunoblotting as described under Section 2. Upper photo shows PPARγs. The larger band is PPARγ2 and the smaller band is PPARγ1. The lower photo shows β-actin. (D) Relative levels of PPARγs in each period. The relative PPARγs levels were normalized with β-actin. 0.1% DMSO (○); 100 nM 9-*cis*-RA (●). The results were repeated a minimum of 3 times.

2.5. Statistical analysis

All data were analyzed using Student's *t*-test. Differences were considered to be statistically significant if the *p*-value was <0.05.

3. Results

3.1. 9-cis-RA suppresses lipid accumulation during adipogenesis

Initially, we added 9-*cis*-RA to the culture medium of DIM-stimulated 3T3-L1 cells to examine the effects of 9-*cis*-RA on the adipogenesis of 3T3-L1 cells (Fig. 1A). The lipid bodies of the cultured cells were stained with Oil Red O, and sub-quantitative estimations of accumulated lipid bodies were determined by measuring the amount of Oil Red O absorbed under each culture-condition. 9-*cis*-RA showed significant suppression of lipid accumulation in dose-dependent manner (Fig. 1B). Lipid bodies were decreased by 7% with treatment of 9-*cis*-RA during adipogenesis at a concentration of 100 nM.

3.2. 9-cis-RA decreases intracellular levels of both RXRα and PPARγ

Next, we assessed RXRα and PPARγ levels of 3T3-L1 cells during adipogenesis. This was done because these transcription factors form hetero-dimer complexes that are mainly responsible for the adipogenesis process. Both RXRα and PPARγ levels were increased in DIM-stimulated 3T3-L1 cells during the course of culturing up to 5 days after DIM stimuli. However, 9-*cis*-RA treatment inhibited increases in both RXRα and PPARγ levels (Fig. 2). RXRα from whole lysates was detectable in non-stimulated cells (Fig. 2A, NT), and DIM-stimuli increased by a factor of 2.1 after 20 h (Fig. 2B, DIM) and by a factor of 8.9 after 3 days (Fig. 2B, 3d) as compared with control (Fig. 2B, NT). 9-*cis*-RA treatment suppressed the increase in intracellular RXRα levels (Fig. 2A and B).

PPARγs of whole lysate, including PPARγ1 and PPARγ2, were also increased by DIM-stimuli 2.2-fold and 6.8-fold, respectively (Fig. 2D, DIM) as compared with control (Fig. 2D, NT). Both PPARγ1 and PPARγ2 showed enhancement in their intracellular levels from 1 day after DIM addition. 9-*cis*-RA treatment suppressed the enhancement of intracellular PPARγs (Fig. 2C and D).

3.3. 9-cis-RA's inhibitory effects on adipogenesis is dependent on RXR activity

9-*cis*-RA is known to be a ligand that binds both RARs and RXRs. In our experiments using Oil Red O staining, the RXR antagonist, PA452, but not the RAR antagonist LE540, partially disturbed the suppressive activities of 9-*cis*-RA on adipo-maturation of 3T3-L1 cells (Fig. 3). PA452 was antagonistic against 9-*cis*-RA. While 100 nM 9-*cis*-RA suppressed the accumulation of lipid bodies in 3T3-L1 cells by approximately 5.3%, 10 μM PA452 had an inhibitory effect (approximately 34.0%) on the suppression of lipid body accumulation induced by 9-*cis*-RA. The inhibitory effects of PA452 were dose-dependent (Fig. 3). These results indicate that 9-*cis*-RA could suppress activity through RXRs.

3.4. 9-cis-RA's inhibitory effects on adipogenesis are independent of RXR, RAR and PPARγs levels

We speculated that PA452 antagonized 9-*cis*-RA's suppressive effects on DIM-induced adipogenesis through increases in RXRα and PPARγs. At 100 nM concentration, 9-*cis*-RA's suppressive effects on the intracellular levels of both RXRα and PPARγs were not hindered by 10 μM of either PA452 or LE540 (Fig. 4A–D). Contrary to the effects observed with 9-*cis*-RA, the RXR agonist, HX630 (100 nM) increased the intracellular levels of PPARγs (Fig. 4C and D), but not RXRα (Fig. 4B). These results indicate that in affecting the lipid accumulation of differentiating 3T3-L1 cells, 9-*cis*-RA acts as a RXR ligand, but not as a RAR ligand, and that the 9-*cis*-RA-induced decreases in both RXRα and PPARγs were independent of RXR activation.

4. Discussion

Our current work demonstrates that 9-*cis*-RA displays suppressive effects on adipocyte maturation in 3T3-L1 cells (Fig. 1). 9-*cis*-RA suppressed the enhancement of RXRα and PPARγ expression in DIM-stimulated 3T3-L1 cells (Fig. 2). The anti-adipogenic activity of 9-*cis*-RA was diminished by an RXR antagonist PA452, but not by the RAR antagonist LE540 (Fig. 3), while the reductions of RXRα and PPARγ expression by 9-*cis*-RA were not recovered by the treatment with RXR and RAR antagonists (PA452 and LE540) (Fig. 4). These results suggest that 9-*cis*-RA reduces lipid accumulation of preadipocytes, and that 9-*cis*-RA could potentially function as an anti-adipogenetic reagent through both RXR-dependent and independent mechanisms.

It is of note that Canan et al. have previously reported [6] that synthetic oxime derivatives of carbonylbenzoic acids, which functioned as RXR-specific agonists, could act as inducers of 3T3-L1 adipogenesis by the activation of RXR-PPARγ heterodimers. ATRA is a specific ligand for RARs, and 9-*cis*-RA is an agonist for both RARs and RXRs. Accordingly, we surmised that 9-*cis*-RA, unlike ATRA, might serve as an agonist of RXR and enhance adipogenesis. However, 9-*cis*-RA suppressed adipogenesis in 3T3-L1 preadipocytes (Fig. 1). We were interested in the mechanism of the suppressive effects of 9-*cis*-RA on adipogenesis. In the adipogenesis model with 3T3-L1 cells, the DIM mixture in the induction medium stimulates adipocyte maturation, and up-regulates transcription fac-

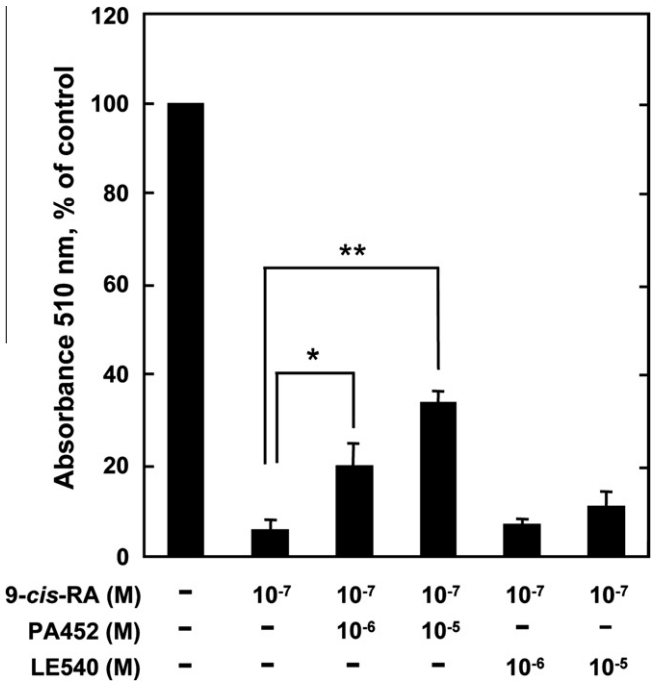

Fig. 3. Effects of retinoid receptor antagonists on adipogenesis by 9-*cis*-RA in 3T3-L1 cells. During the adipogenesis process, DIM-stimulated 3T3-L1 cells were treated with 100 nM 9-*cis*-RA ± PA452 (RXR antagonist) or LE540 (RAR antagonist), and 100 nM HX630 (RXR agonist) for 7 days. At day 7 following retinoid-addition, the accumulation of lipid bodies was evaluated with Oil Red O staining as described under Section 2. Each bar represents the mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control compared by Student's *t*-test.

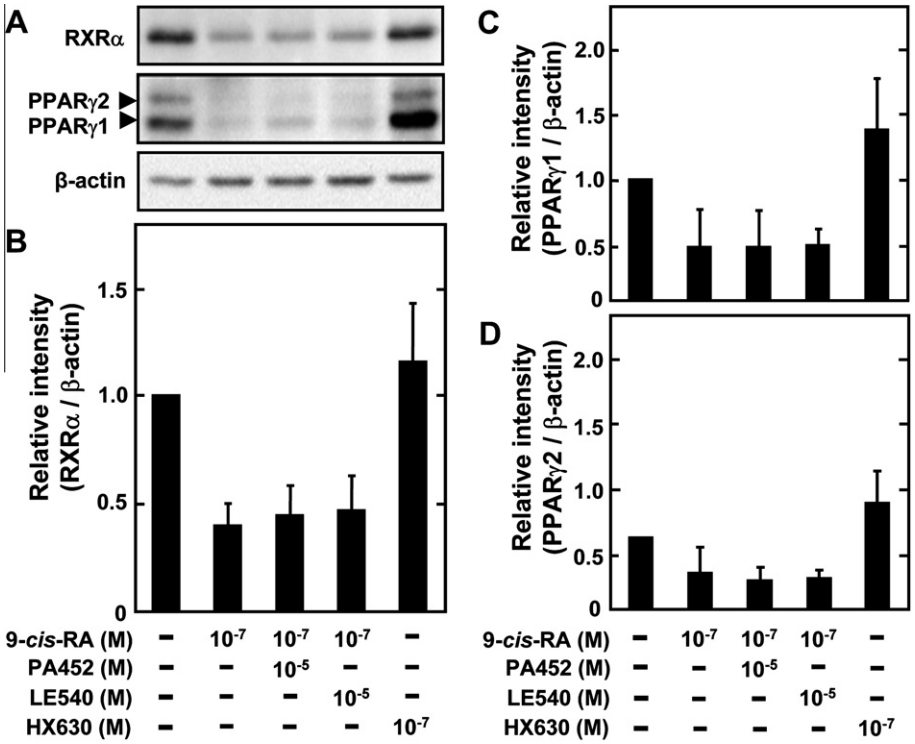

Fig. 4. Effects of retinoid receptor antagonists on the intracellular RXRα and PPARγs levels in the suppression of adipogenesis by 9-*cis*-RA in 3T3-L1 cells. (A) During the adipogenesis process, DIM-stimulated 3T3-L1 cells were treated with 100 nM 9-*cis*-RA ± PA452 (RXR antagonist) or LE540 (RAR antagonist), and 100 nM HX630 (RXR agonist) for 7 days. At day 7 following retinoid-addition, whole lysates were prepared. RXRα and PPARγs were detected with immunoblotting as described under Section 2. Experiments were performed at least 3 times. B–D show the graphs of relative levels of RXRα, PPARγ1 and PPARγ2, respectively, normalized against β-actin. Each bar represents the mean ± SD. $^{*}p < 0.05$ versus control compared by Student's *t*-test.

tors, including PPARγ. As shown in Fig. 2C and D, intracellular PPARγ1 and γ2 levels were increased by DIM-stimuli and were enhanced from 1 day after DIM treatment. 9-*cis*-RA suppressed the enhancement in the intracellular levels of both PPARγ1 and PPARγ2. Inhibitory effects of 9-*cis*-RA on intracellular levels of RXRα were also observed, with enhancement at 3 days being completely suppressed by 9-*cis*-RA treatment (Fig. 2A and B). 9-*cis*-RA treatment could potentially induce the inactivation of RXR-PPARγ heterodimers by decreasing both PPARγs and RXRα during the adipogenetic process.

As shown in Fig. 2B, intracellular RXRα levels changed in a two-phase pattern. The first phase occurred 24 h after DIM-stimuli, when RXRα levels increased ~3 times more than NT levels, and the second phase was observed after 3 days, when the level of RXRα was enhanced ~8-fold greater than NT levels. 9-*cis*-RA treatment did not increase intracellular RXRα protein levels (Fig. 2A and B). RXRα containing phospho-serine260 RXRα is resistant to ubiquitination and proteasome-mediated degradation in various cell types [23–25]. DIM-enhanced kinase activities might increase phosphorylation of biosynthesized RXRα and thereby result in the accumulation of phospho-RXRα, which could modulate adipogenesis through gene expression. 9-*cis*-RA might decrease the levels of phosphorylated RXRα through inhibition of kinases or activation of phosphatases. From semi-quantitative RT-PCR analysis, *rxra* transcripts in DIM-stimulated 3T3-L1 cells were down-regulated at 8 h following addition of 9-*cis*-RA (data not shown). The suppressive effects of 9-*cis*-RA on RXRα levels could be caused not only by post-translational modification but also by transcriptional synthesis.

In 3T3-L1 preadipocytes, effects of 9-*cis*-RA were also observed on PPARγs protein levels (Fig. 2D). Intracellular PPARγs levels in 3T3-L1 cells were significantly increased at 1 day following DIM-stimuli (Fig. 2D). 9-*cis*-RA treatment suppressed the increase in PPARγs levels. It should be noted that *ppargs* transcripts in DIM-stimulated 3T3-L1 cells were not influenced by 9-*cis*-RA treatment (data not shown). These results suggest that 9-*cis*-RA might promote proteolysis of PPARγs.

9-*cis*-RA is known as a ligand of both RARs and RXRs. In the current study, the RXR antagonist PA452, but not the RAR antagonist LE540 partially, disturbs the suppressive activities of 9-*cis*-RA on adipogenesis of 3T3-L1 cells (Fig. 3). In 3T3-L1 cells 9-*cis*-RA was able to suppress lipid accumulation during adipogenesis by acting through RXRs, rather than through RARs. We speculated that PA452 could antagonize 9-*cis*-RA's suppressive effects on DIM-induced adipogenesis by increasing RXRα and PPARγs levels. However, in 3T3-L1 cells the suppressive effects of 100 nM 9-*cis*-RA on the intracellular levels of both RXRα and PPARγs were not hindered by 10 μM PA452 and 10 μM LE240 (Fig. 4). Decreases in both RXRα and PPARγs following 9-*cis*-RA treatment were independent of RXR activation.

A fundamental function of retinoids is to act through transcriptional regulation by modulating gene expression in a hormone-like fashion. This occurs by binding to specific nuclear receptors, RAR and RXR. However, a variety of cellular effects of retinoids have recently been identified that may be unrelated to their receptor binding interactions. Retinoids can participate in post-translational protein modification through retinoylation. Takahashi et al. have shown that enzymatic retinoylation systems exist *in vivo*, and that the substrates for retinoylation reactions are regulated by cellular responses toward retinoids [26–29]. During adipogenesis in 3T3-L1 cells, lowering of PPARγs and RXRα appears to be independent of RXR activation, and instead 9-*cis*-RA may function as a post-translational modifier of proteins that include RXRs and PPARγs.

Acknowledgments

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