TITLE: Retinoic Acid modulates *PTGDR* promoter activity

CONDENSE TITLE: RA and *PTGDR* gene

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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ABSTRACT

Background

Vitamin A has been involved in the development of allergic diseases although its role is not fully understood. Retinoic Acid (RA), a metabolite of Vitamin A, has previously been associated to the prostaglandin pathway. In addition PTGDR, a receptor of PGD2, has been proposed as a candidate gene in allergy and asthma.

Objective

Considering the role of PTGDR in allergy, the goal of this study was to analyze the effect of the retinoic acid over the activation of the promoter region of *PTGDR* gene.

Methods

A549 lung epithelial cells were transfected with four combinations of genetic variants of *PTGDR* promoter and stimulated with all-trans Retinoic Acid (ATRA). Luciferase assays were performed by Dual Luciferase Reporter System. RT-qPCR was used to measure the *PTGDR*, *CYP26A1*, *RARA, RARB, RARG* and *RXRA* basal expression of A549 cell cultures and after ATRA treatment. *In silico* analysis was also performed.

Results

After ATRA treatment an increase of the expression of *CYP26A1* (12 fold) and *RARB* (4 fold) was detected. ATRA activated the *PTGDR* promoter activity in transfected cells ($p<0.001$). Retinoic Acid Response Elements (RARE) sequences were identified "*in silico*" in this promoter region.

Conclusion & Clinical Relevance

Retinoic acid modulated *PTGDR* promoter activity. Differential response to RA and to new treatments based on PTGDR modulation could depend on the genetic background in allergic asthmatic patients.

KEY WORDS: Allergic Asthma; Polymorphisms; *PTGDR*; Retinoic Acid.

RESUMEN

Introducción

La vitamina A se ha relacionado con el desarrollo de las enfermedades alérgicas,si bien su papel no ha sido comprendido totalmente. El ácido retinoico (RA), un metabolito de la vitamina A, se ha asociado previamente con la ruta de las prostaglandinas. Además PTGDR, uno de los receptores de PGD2, se ha propuesto como un gen candidato en la alergia y el asma.

Objetivo

Considerando el papel de PTGDR en la alergia, el objetivo de este estudio fue analizar el efecto del ácido retinoico sobre la activación de la región promotora del gen *PTGDR*.

Material y Métodos

Se utilizó la línea celular A549 de epitelio de pulmón. Las células fueron transfectadas con cuatro combinaciones de las variantes génicas de *PTGDR*y fueron estimuladas con Ácido Retinoico todo-trans (ATRA). Los ensayos de Luciferasa se llevaron a cabo mediante el sistema Dual Luciferase Reporter System. Se realizaron análisis de RT-qPCR para medir la expresión basal de *PTGDR*, *CYP26A1, RARA, RARB*, *RARG y RXRA*de los cultivos de A549 tras el tratamiento con ATRA. Se realizaron también análisis bioinformáticos.

Resultados

Se encontraron diferencias significativas en la actividad promotora entre las variantes haplotípicas tras la transfección en la línea celular A549. Tras el tratamiento con ATRA se detectó un incremento de la expresión de *CYP26A1* (12 veces) y*RARB* (4 veces). El ácido retinoico activó la actividad promotora de *PTGDR* en las células transfectadas

(p<0.001).Se identificaron secuencias de Elementos de Respuesta a Ácido Retinoico (RARE) *in silico* en la región promotora de *PTGDR*.

Conclusión y Relevancia Clínica

El Ácido Retinoico modula la actividad promotora de *PTGDR*. Esto podría explicar las diferencias en los efectos del ácido retinoico y en las respuestas a los nuevos tratamientos de la enfermedad alérgica basados en la modulación del receptor PTGDR

Palabras clave: Asma alérgica; Polimorfismos; *PTGDR*; Ácido Retinoico.

INTRODUCTION

Asthma and allergic diseases are believed to be the result of the interaction between multiple genes and environmental stimuli. Several features associated with a westernized lifestyle have been suggested as possible causes of the increased prevalence of allergy, among others an increased exposure to indoor allergens, changes in the exposure to contaminants and pollutants, a reduced rate of infectious diseases, or a Western diet [1]. A westernized diet is associated to a decreased intake of fruits and vegetables (sources of antioxidant vitamins and carotenoids) leading to decreased intakes of vitamins E and A. Epidemiological studies have reported results regarding the association of vitamin A and allergy and several studies indicated that vitamin A affects the Th1/Th2 balance [2, 3]. The effect of vitamin A has been studied in OVA-induced allergic mouse models of asthma [4] and it has been described to play an important role in Treg differentiation and function [5], although the underlying mechanisms are not fully understood. The role of ATRA in asthma and allergic disease is controversial, while some studies found vitamin A deficiency (VAD) to be associated with increased risk of atopic disease [6], other studies reported that VAD may be protective against asthma and vitamin A supplementation (VAS) is associated with increased airway hyper-responsiveness [4].

ATRA is a biologically active metabolite of vitamin A that exerts profound effects on T-cell activation, differentiation, and function [7]. ATRA regulates gene transcription mainly through two families of nuclear receptors, the retinoic acid receptors (RAR-α,-β,-γ) and the retinoid X receptors (RXR-α,-β,-γ) [8], and, under certain circumstances, PPARβ [9].

Effects of retinoic acid over prostaglandin pathways have also been reported. ATRA increased the expression of COX-2 and the production of PGE2 [10]. The biosynthesis

of prostaglandin D2 is mediated by two isoforms, Lipocalin-type Prostaglandin D Synthase (LPGDS), present in the central nervous system and the genital organs, and the hematopoietic-isoform (HPGDS), present fundamentally in mast cells and Th2 lymphocytes [11]. The LPGDS is a retinoid transporter capable of binding ATRA [12]. The prostaglandin D2 is the most abundant cyclooxygenase metabolite of arachidonic acid produced by mast cells in response to environmental allergens [13]. Prostaglandin D2 exerts its biological actions through the specific transmembrane receptors, PTGDR and CRTH2 [11]. The association between allergy and *PTGDR* promoter polymorphisms has been previously reported [14-16]. It has been shown that the capacity of the *PTGDR* promoter regions to bind transcription factors, and therefore to control the *PTGDR* expression, appears to be associated to the development of susceptibility to the disease [14-16]. Considering that, our hypothesis is that a possible role of RA in the regulation of the immune response could be involving PTGDR. The objective of this study was to analyze the effect of RA over the activation of the promoter region of *PTGDR*.

METHODS

Cell culture

The A549 epithelial lung cell line was kindly provided by Dr. MD Odero (CIMA, University of Navarra) and maintained in RPMI with 10% FBS (Gibco, Invitrogen-LifeTechnologies, CA, USA) in a humidified atmosphere with 5% CO_2 at 37ºC. This lung cell line is the model previously used in *PTGDR* expression analysis in susceptibility to asthma studies [14].

Reagents

ATRA stock solutions were made in DMSO (both from Sigma-Aldrich, St. Louis, MO, USA), and added directly to the cell culture media to produce the final concentration.

Identification of A549 sequence variants

Genomic DNA from A549 cell line was isolated using the MagNA Pure Nucleic Acid Isolation Kit in the MagNA Pure Compact instrument (Roche, Mannheim, Germany). Genomic DNA was screened for mutations in the promoter region of *PTGDR* by PCR using the oligonucleotids primers sense 5'-CTC AGT TTC CTC GCC TAT GC-3' and anti-sense5'-GAG TGA AGG CTG CGG AAG GG-3'. PCR products were treated with exoSAP-IT (USB-Affimetrix, Santa Clara, CAL, USA) for cleaning up until sequencing in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems-Thermo Scientific, Whaltham, MA, USA).

Plasmids preparation

Four *PTGDR* reporter constructs of 700 bp were created by PCR amplification of genomic DNA from asthmatic patients who had the following alternative homozygous haplotypes (positions -613, -549, -441 and -197): CTCT, CCCT, TCCT and CCCC; with the use of primers that included the *XhoI* and *BglII* recognition sites. The selected 700 bp sequence comprises the four most studied variants so far and the combinations selected were the most frequently observed in our patients with asthma, in order to

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design a model close to the clinical context [15, 16]. The rest of the sequence was in wild type conformation. Amplicons were cloned into the plasmid pSC-A-amp/kan of StrataClone (Stratagene, Agilent Technologies, CA, USA). The constructs generated were subcloned and ligated into multicloning sites of the firefly luciferase pGL3-basic vector (Promega, Wisc., USA). Plasmid DNA was purified with Maxiprep (Qiagen, Hilden, Germany). The pGL3-basic vector was also a kind gift from Dr. MD Odero.

Real-Time Quantitative PCR (RT-qPCR)

PTGDR, *CYP26A1*, *RARA*, *RARB*, *RARG* and *RXRA* expression analysis was also performed in basal human A549 cell cultures and in cells treated with 0.01 μM, 1 μM ATRA or DMSO as vehicle and incubated during 24 and 48 h. Total RNA was isolated using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNAse treatment was performed using Turbo DNAse (Ambion, TX, USA). cDNA was generated using Superscript III (Life Technologies, Paisley, UK). Relative quantitative PCR was performed using LightCycler 480 Instrumente using SYBR Green I Master (Roche, Basel, Switzerland). Fold induction was calculated using the formula $2^{-(\Delta\Delta Ct)}$. PCR product quality was monitored using post-PCR melt curve analysis. Data are expressed as fold change relative to RNA levels for DMSO treated cells. The experiments were performed in triplicate.

Transient Transfections and Luciferase Assays.

A549 transient transfection was carried out with Lipofectamine reagent 2000 (Invitrogen, CA, USA) according to the manufacture's protocol. Fifty to seventy percent confluent cells seeded on 24-well plates were transfected with 500 ng of reporter plasmid (pCTCT-*PTGDR*luc, pCCCC-*PTGDR*luc, pCCCT-*PTGDR*luc and pTCCT-*PTGDR*luc) and cotransfected with 10 ng of the PRL SV 40 vector (Renilla) (Promega, Madison, WI, USA) to monitor transfection efficiency. After five hours the transfection

medium was remove and replaced by fresh medium with antibiotics. Twelve hours after transfection, cells were lysed. The Firefly and Renilla luciferase activities were measured in a LumiStar Omega Luminometer (BMG LabTech, Germany) using Dual-Luciferase Reporter Assay System (Promega, WI, USA). The transfection efficiency was corrected by normalizing the Firefly luciferase activity to the Renilla luciferase activity. Each assay condition was performed in 3 replicates. Reporter activity was expressed as mean ± S.D.

RA treatment in transient transfections

Cells were seeded in RPMI serum free medium 24 h before the transfection assay. After five hours of the above-mentioned transient transfections the transfection medium was replaced by RPMI supplemented with 1% FBS and cultures were treated with 1 μM ATRA or DMSO as vehicle and incubated for 12 and 48 h. Cells were lysed and Firefly and Renilla luciferase activities were measured as previously mentioned. The experiments were replicated 3 times.

***In silico* characterization of transcription factors**

To identify the presence of putative binding-sites for transcription factors, a bioinformatics analysis in the promoter region of the *PTGDR* gene was performed. The analysis included a 1,000 bp region just before the transcription start site. *In silico* analysis were carried out using the MatInspector (www.genomatix.de) and Match-BioBase programs (http://portal.biobase-international.com/).

Statistical analysis

All data were presented as mean ± SE. The comparison between data were performed using unpaired one-sample t-test using SPSS Software (version 17.0). Data were

representative of at least three independent experiments. $P<0.05$ was considered significant.

RESULTS

A549 cell line does not express *PTGDR* in basal conditions

We analyzed the basal expression of *PTGDR* gene in A549 cell line and mRNA expression was not detected. We genotyped the *PTGDR*promoter region of A549 human lung cells to identify the genetic variants, resulting the CTTT haplotype (-613 C, -549 T, -441 T and -197 T).

In addition, we studied the mRNA expression of genes that codify for the retinoic nuclear receptors RARα, RARβ, RARγ, RXRα and the CYP26A1 RA metabolizer enzyme. In basal conditions expression of these genes was not detected.

Genetic variants regulate the promoter activity driven by the 5'-flanking region of *PTGDR.*

To interrogate the contribution of *PTGDR* SNPs to *PTGDR* expression in cell culture, a 700 bp fragment upstream of the transcription start site (TSS) of human *PTGDR* was amplified and cloned in the pGL3-basic vector. The fragments were obtained from asthmatic patients to put the model in the physiological context. Four asthmatic patients carried the more frequent haplotypic variants (positions -613, -549, -441 and -197) CTCT, CCCC, CCCT or TCCT were selected. The cloned regions were confirmed by sequencing.

We found significant differences in the promoter activity among the haplotypic variants after transient transfection analysis in the A549 cell line (Figure 1 A). The haplotype with the wild type CTCT sequence (-613 C, -549 T, -441 C, and -197 T), CCCT and TCCT showed the lowest reporter activity, while the other haplotype, CCCC, showed a highly remarkable reporter activity.

The promoter activity after normalization (mean±SD) was expressed as the Relative Luciferase Units (RLU), considering the sylvestre variant CTCT as value 1 CCCC: 1.22±0.08; CCCT: 0.92±0.12; TCCT: 0.97±0.04 RLU, (p-value<0.001, for all pairwise comparison by analysis of variance, except for the comparison of CCCC with CTCT, for which p<0.05) (Figure 1 A).

Interestingly, all the haplotypic combinations with high level of *PTGDR* promoter activity differed from the lowest reporter activity by carrying the C mutant variant at the -197 promoter position. No differences were observed between the non-transfected and the cells transfected with the non-mutated CTCT combination.

Retinoic Acid selectively stimulates gene expression in A549 cells

To deeper insight into the retinoic acid regulation mechanisms, the expression of genes coding CYP26A1 (enzyme that regulates retinoic acid levels), and the retinoic receptors RARα, RARβ, RARγ and RXRα was analyzed in A549 cells after stimulation with 0.01 and 1 μM ATRA during 24 and 48 h in order to analyze dosis-response and time-course gene expression (Figure 2).

Elevated levels of *CYP26A1* (12 fold) and *RARB* (4 fold) were detected after 1 μM ATRA treatment, confirming the activation of the retinoic pathway. This increase was maintained until 48h after ATRA stimulation. We did not detect any expression of *RARA*, *RARG* nor *RXRA* genes in the treated A549 cell line.

In addition, *PTGDR* mRNA was analyzed in these cells after ATRA stimulation and no expression was detected.

Retinoic acid stimulates the promoter activity driven by the 5'-flanking region of *PTGDR*.

To determine whether ATRA increases the *PTGDR* promoter activity, we examined the transcriptional activity of the 5'-flanking region of *PTGDR* (Figure 1 B) using the dual-luciferase reporter assay method normalized to cells treated with DMSO (value 1). Since the therapeutic level of ATRA in human plasma is about 1-2 μM [17], we selected the concentration of 1μM for luciferase experiments. When stimulated with ATRA at 12 and 48 h, all haplotypic variants exhibited higher activity ($p<0.001$). These results were confirmed in serum-free experiments to avoid the possible effect of serum components.

Transcription Factor Binding Site Analysis in the 5'-Flanking Region of *PTGDR*

To deeper inside the activation mechanism in the promoter region of *PTGDR* different bioinformatic analysis using MatInspector and BioBase programs were developed. We identified for the first time putative Retinoic Acid Response Elements sequences by the two platforms employed in this promoter region.

In addition, the transcription factor motifs were affected by the promoter variants. *In silico* studies revealed putative binding differences between wild and mutated alleles in -613, -549 and -197 position. Interestingly, these changes affected the binding affinity ofNF-AT, PAX6, EST-1, PPAR, RAR, RXR, GZF1, SORY, AP-1, NBRE, ZNF652, MAZF and SRFF transcription factors.

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DISCUSSION

A549 cell line does not express *PTGDR* in basal conditions

Human *PTGDR* promoter region is known to exhibit genetic variability, which has a considerable importance in asthma studies [14-16]. To check our hypothesis based on a possible retinoic effect over *PTGDR* promoter activation we selected a cell lung model previously used in *PTGDR* expression studies of susceptibility to asthma [14]. The *PTGDR* expression in A549 at basal conditions was undetectable confirming that this was an ideal model for analyzing the functional effects of combination of individuals *PTGDR* variants. Sequencing revealed that the A549 epithelial cell lung used in our experiments has the haplotype -613 C, -549 T, -441 T and -197 T, supporting the hypothesis that haplotypic combinations carrying the allele T at -197 position influence negatively in transcriptional regulation of *PTGDR* [14].

Genetic variants regulate the promoter activity driven by the 5'-flanking region of *PTGDR*

In this study, after transient transfection, genetic variants that included the mutated C nucleotide in the -197 *PTGDR* promoter position had remarkable higher promoter activation in cell culture. Expression of genetic variants has been described in previous models including three polymorphic positions [14]. We included the analysis of the -613 position, which has been also related to asthma in genetic association studies [15, 16].

The CTCT wild type *PTGDR* haplotype (-613 C, -549T, -441C, -197T) has been detected more frequently in controls than in allergic subjects in a previous report [18]. In our study the CCCC haplotype significantly associated with the highest *in vitro* reporter activity, while the rest of haplotypes, CTCT, CCCT and TCCT, showed a

significant lower *PTGDR* promoter activation.The CCCC haplotype contains CCC (-549, -441, -197), a combination described as a high transcriptional efficiency haplotype related to *PTGDR* expression and previously associated to predisposition to asthma [14]; Although other SNPs or combinations can be responsible of variation of *PTGDR* expression, according to our results the haplotypic combination exert more effect than individual positions.

Retinoic Acid selectively stimulates gene expression in A549 cells

We interrogate expression of both RXR and RAR receptors in non-transfected A549 cells and after ATRA stimulation and interestingly we detected a selective increase of retinoid receptors after ATRA treatment. RA regulates inflammation, cell proliferation and differentiation, tissue development and maintenance, and immune homeostasis, modulating the target gene transcription ([7, 10, 19, 20]. Its activity is mediated by RXR, and RAR receptors. RAR isotypes differ in their NH2-terminal domain as a result of alternative promoter usage and splicing. It has been suggested that interaction between retinoids and some of their receptors overexpressed by the bronchial epithelium of patients with severe asthma, may contribute to an abnormalrepair and to airway remodeling [21]. In our study only RARB expression was detected. It has been reported that RARB expression appears to be largely dependent on RA itself and, therefore, may play a prominent role in the regulation of immune responses [22]. In addition, we detected elevated levels of *CYP26A1* gene. CYP26A1 monooxygenase inactivates ATRA by its 4-hydroxylase activity. Over more, *CYP26A1* is also inducible by ATRA through RARE elements in the *CYP26A1* promoter region [23]. We previously reported different epigenetic patterns in *CYP26A1* in allergic patients [24]. Both

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CYP26A1 and *RARB* have been found to be greatly inducible by ATRA in a hepatic cell model. Here we showed similar effects in an epithelial lung model.

Finally, we did not detect expression of *PTGDR* after ATRA treatment. We would like to highlight that these cells carry the variant with the allele T at -197 position, which appears to be resistant to the retinoic acid effect.

Retinoic acid stimulates the promoter activity driven by the 5'-flanking region of *PTGDR*.

We describe for the first time that RA stimulates the promoter activity driven by the 5'-flanking region of *PTGDR*. Retinoid bind to specific RAREs in the promoter regions of target genes and function as inducible ligands by transcription factors [8]. RA has previously been associated to the prostaglandin pathway. PGD_2, is the most abundantly produced cyclooxygenase metabolite of arachidonic acid in response to allergens and a mast cell activation marker in asthma [25]. PGD_2 is synthesized by COX-1, COX-2, HPGDS and LPGDS. It has been shown that ATRA increased the expression of COX-2 [26]. The LPGDS is a binding ATRA transporter [12] and its over-expression increased the concentration of Th2 cytokines [27]. In addition, Su et al. showed that RA modulates cell proliferation in ovarian tumor through LPGDS [28]. We not only detected an activation of promoter region of *PTGDR* after retinoic treatment, we also described that this activation depends on the presence of promoter combinations although the differences did not reach statistical significance. Activation of *PTGDR* by retinoic acid could have important implications in the immune modulation and open new insight in the field. In addition, the selective effect could explain certain discrepancies related to retinoic effects on allergic patients.

Transcription Factor Binding Site Analysis in the 5'-Flanking Region of *PTGDR*

To interrogate the probable mechanisms underlying this selective promoter activation we developed an *in silico* analysis and we found potential RAREs sequences in the *PTGDR* promoter. In addition, the *in silico* analysis of putative binding sites showed that differential allelic occupancy at -613, -549 and -197 position determines modifications in transcription factors binding that could mediate the differential promoter activation. In this study only the mutated variants that bind PAX6 and ETS-1 and ZFN652 in the -549 position and bind MYC and SRF in the 197 position seemed to activate the *PTGDR* promoter, so we could infer that these or other still non identified TFs could be necessary to activate *PTGDR* expression. ETS-1 is involved in allergen-challenged CD4+ cells and siRNA-mediated knockdown of ETS-1 resulted in modification of Th2 cytokines levels [29]. NF-AT develops a key play role in the transcription of cytokine genes and other genes during the immune response. NF-AT binds to the promoter of *IL2, IL4, IL5, IL13, GM-CSF* and *TNFA* [30, 31]. In addition, ZNF652 encodes a transcription factor that has been identified in a GWAS of atopic dermatitis in European and Asian populations [32]. MYC, a transcription factor involved in multiple cellular processes has been also identified in the first large-scale GWAS of allergic sensitization [33]. Meanwhile, SRF is involved in airway remodelation in asthma acting as cofactor in association with SMAD to activate the TGFβ signalling pathway [34].

The RA role in allergy is not fully understood. It supports the differentiation and functional maturation of innate immune cells although how they contribute to T cell activation is still unclear [7]. RA regulates the Th1/Th2 balance [2] and particularly the Th2 regulation by NF-AT transcription factors [35]. Future Electrophoretic Mobility

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Shift Assay (EMSA) and Chromatin Immunoprecipitation (ChIP) assay experiments should be conducted in order to confirm these interesting results.

Epidemiological studies have reported both direct and inverse association between asthma and the intake of vitamin A [2, 3, 4, 6]. Potential benefits of therapeutic effects of ATRA or vitamin A in controlling the airway inflammation of asthma may provide the basis for further investigation.

The majority of the susceptibility studies are based only on descriptive analysis, which confers an important limitation to provide information on the mechanism by which the variant leads to the disease [14]. Our results indicate a selective respond of epithelial lung cells to retinoic treatment highlighting the role of the RAR β isoform. In addition, we describe for the first time an effect of retinoic acid over activation of the promoter region of *PTGDR*. We also showed a selective respond according to the genetic variants, although statistical significance was not reached. Over more, we identified RARE elements in this promoter region detecting that differential allelic occupancy at different positions determines modifications in transcription factors binding that could explain differential promoter activation. All these results provide new insights to retinoic acid effects and to treatments based on *PTGDR* modulators [36] although more studies are needed to assess the relevance of these results to allergic diseases.

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FIGURE LEGENDS

Figure 1. **Transcriptional activity and ATRA modulation of the 5'-flanking region of *PTGDR*. A)** A549 cells were transfected in triplicate with PTGDRluc reporter plasmid bearing the the CTCT (wt) or CCCC, CCCT, TCCT (mutated) *PTGDR* promoter sequences. Graphic shows the promoter activity of different haplotype variants. Each value represents mean±SD of relative light activities to pCTCT-PTGDRluc vector activity (* $p<0.05$, ** $p< 0.01$, n=3). **B)** A549 cells were transfected in triplicate with PTGDRluc reporter plasmid bearing the the CTCT (wt) or CCCC, CCCT, TCCT (mutated) *PTGDR* promoter sequences. After transfection, cells were stimulated with 1 μM ATRA for 12 and 48 h. Each value represents the relative promoter activities mean±SD of light activities of ATRA relative to DMSO (value 1) (***$p<0.001$, n=3).

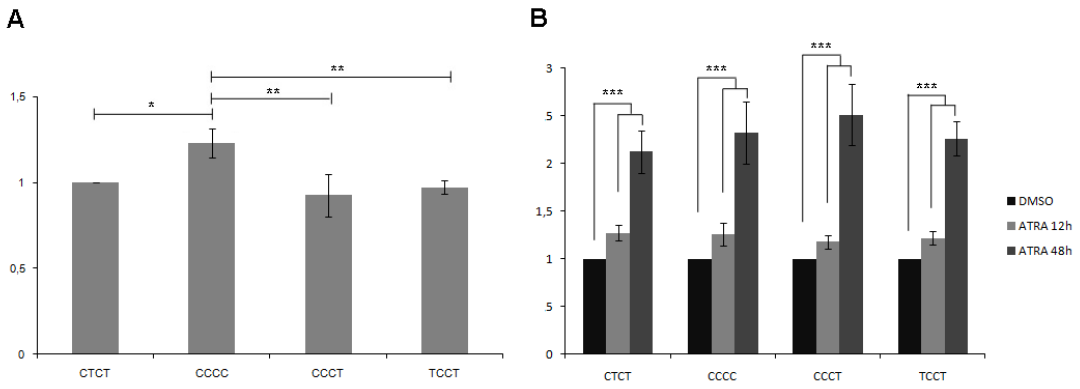

Figure 2. **Quantitative real-time PCR analysis of *CYP26A1* and *RARB* gene expression induced by ATRA.** A549 cells were treated with 0.01 and 1 μM for 24 and 48h followed by real-time PCR analysis of *CYP26A1* and *RARB* mRNAs. mRNA levels were normalized relative to *GAPDH* mRNA levels. Data are shown as fold increase relative to mRNA levels for DMSO treated cells at the same times.