



# Differential and bi-directional regulation between TR2/TR4 orphan nuclear receptors and a specific ligand mediated-peroxisome proliferator-activated receptor $\alpha$ in human HaCaT keratinocytes

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## KEYWORDS

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**Summary Background:** We have reported that human TR2 orphan nuclear receptor (TR2) can modulate the transcriptional activity of the reporter gene containing an AGGTCA direct repeat-hormone response element. **Objective:** The aim of this study is to investigate the potential role and regulation of TR2 in human HaCaT keratinocytes. **Methods:** We performed mainly chloramphenicol acetyltransferase reporter gene assays (CAT assays), and Western blot analysis. **Results:** From CAT assays, TR2 can suppress retinoic acid (RA)-induced transactivation by 44.7% in HaCaT keratinocytes. This suppression is similar to our previous report showing TR4 orphan nuclear receptor (TR4) can suppress RA-induced transactivation. However, TR4 but not TR2 can significantly repress Wy-14643-mediated peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) transactivation by 95%. Western blot analysis suggested that Wy-14643 can differentially regulate the expression of TR2 and TR4 (by increasing the expression of TR4 protein and decreasing that of TR2) in HaCaT keratinocytes. **Conclusion:** Our data not only provides the first evidence to demonstrate that close members of orphan nuclear receptors group, such as TR2 and TR4, can have distinct functions, but also suggests the existence of differential and bi-directional regulation between PPAR $\alpha$  and TR2/TR4, that may play some important roles in the PPAR $\alpha$  signaling pathway in human keratinocytes.

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## 1. Introduction

The ligands of some members of the steroid/thyroid hormone receptor superfamily have not been identified. Hence, these receptors are designated as orphan nuclear receptors [1]. The human testicular orphan nuclear receptors, TR2 and TR4, were isolated by our laboratory from human

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prostate and testis [2–4]. From cDNA sequence analysis, the amino acid sequences of TR2 and TR4 are closely related to each other, and it has been shown that they can bind to AGGTCA direct repeats (DR<sub>x</sub>; AGGTCA (n)<sub>x</sub> AGGTCA, x = 0–6) to regulate their target genes [5–11]. Therefore, TR2 and TR4 may form a unique subclass among the steroid/thyroid hormone receptor superfamily.

As TR2 and TR4 have very similar effects on many signaling pathways [5–11], it will be interesting to determine if TR2 and TR4 can have any distinct functions, especially in cell differentiation and/or apoptosis. Indeed, there is evidence that TR2 and TR4 may play important roles in the RA-mediated cell differentiation and apoptosis (Lee and Chang, paper in preparation), and it has been reported that peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) signal pathway can enhance keratinocyte differentiation [12]. In this study, we have focused on the potential cross-talk between TR2/TR4 and PPAR $\alpha$  signaling pathways in HaCaT cells. The PPAR subtypes  $\alpha$ ,  $\delta$  and  $\gamma$  are differentially activated by several fatty acids including arachidonic acid metabolites and hypolipidemic drugs, such as clofibrate. Wy-14643, which we utilized in this study, is a clofibrate analog specific for PPAR $\alpha$ .

We demonstrate here that TR2 and TR4 suppress RA-mediated, but not vitamin D-mediated, transcriptional activity in human HaCaT keratinocytes, using a chloramphenicol acetyltransferase (CAT) reporter gene assay. We also demonstrate that TR4, but not TR2, can significantly suppress the Wy-14643-induced PPAR $\alpha$  transactivation in HaCaT keratinocytes. Furthermore, Wy-14643 can increase TR4 and decrease TR2 expression in HaCaT keratinocytes. These results indicate that a differential and bi-directional regulation between TR2/TR4 and PPAR $\alpha$  can occur in HaCaT keratinocytes, providing the evidence that TR2 and TR4 may play important roles in HaCaT keratinocytes.

## 2. Materials and methods

### 2.1. Cell culture

Human HaCaT keratinocytes (originated in Dr Norbert Fusenig's laboratory), an immortalized cell line, were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and 100 U/ml penicillin/streptomycin [13]. For Wy-14643 and vitamin D treatment, we used medium containing charcoal-stripped fetal calf serum. Keratinocyte cultures were established as described previously [14].

### 2.2. Plasmids

To construct the pCMV-TR2 plasmid, the full-length TR2 cDNA was ligated into the *Sma* I site of pCMV. The pCMX-TR4 plasmid was constructed as described previously [11]. To construct pRAR $\beta$ -CAT, the double-stranded oligonucleotides of retinoic acid (RA) response element (GGTTCacc-gaaAGTTCA) from the promoter region of retinoic acid receptor  $\beta$  (RAR $\beta$ ) were synthesized [15] and ligated into the *Bgl* II site of pCATpromoter vector (Promega). The reporter plasmid P450cc24-CAT, which contains the 5'-flanking region (–2200 to +188) of the rat vitamin D<sub>3</sub> 24-hydroxylase gene, was kindly provided by Dr Kato [16]. PPRE<sub>3</sub>-tk-LUC, the luciferase reporter plasmid containing the peroxisome proliferator response element (PPRE), obtained from the rat acyl-CoA oxidase promoter, was a kind gift from Dr Evans [17].

### 2.3. Coupled in vitro transcription and translation

The full-length TR2 and TR4 were in vitro transcribed and translated by the rabbit reticulocyte-based transcription/translation kit (TNT coupled reticulocyte lysate system) (Promega) according to the manufacturer's instructions.

### 2.4. Transient transfection

Polybrene with DMSO shock was used for transient transfection into HaCaT keratinocytes as reported previously [18]. Four microgram of CAT constructs were co-transfected with 0.01–2  $\mu$ g of pCMV-TR2 or pCMX-TR4. To normalize the transfection efficiency, 1  $\mu$ g of  $\beta$ -galactosidase expression vector, pCMV $\beta$  (Clontech), was co-transfected in all experiments.

### 2.5. CAT assay

After 24 h transfection, cells were treated with  $10^{-6}$  M *all-trans* RA,  $10^{-7}$  M 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>), or ethanol as a vehicle. After 48 h transfection, the cells were washed twice with phosphate-buffered saline and harvested. The cells were lysed by three freeze–thaw cycles and the lysates were centrifuged for 5 min at 14 000 rpm. To inactivate the internal inhibitory fraction for CAT activity, the supernatants were incubated at 57 °C for 10 min and used in CAT reaction. The reaction products were dissolved with ethyl acetate and applied to thin-layer chromatography using a solvent of 95% chloroform and 5% methanol.

CAT activity was measured by PhosphorImager (Molecular Dynamics).

## 2.6. Luciferase assay

After 24 h transfection,  $10^{-5}$  M Wy-14643, a ligand specific for PPAR $\alpha$ , was added into the culture. After 48 h transfection, the cells were harvested and the luciferase activity was assayed according to the manufacturer's instructions (Promega).

## 2.7. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay analysis was performed as previously described [19]. The oligonucleotides were 5' end labeled with  $\gamma$ - $^{32}$ P ATP (specific activity = 6000 Ci/mmol) (New England Nuclear) by T4 polynucleotide kinase. The specific activity reached  $2-8 \times 10^8$  cpm/ $\mu$ g. Labeled oligonucleotides (0.1 ng) were incubated with in vitro translated proteins, in the presence or absence of unlabeled oligonucleotide competitor or rabbit antiserum against TR2 for 15 min at room temperature. The binding buffer contained 25 mM Tris-HCl/pH 8.0, 10% glycerol, 50 mM KCl, 2 mM EDTA, 0.05% Triton X-100, 5 mM DTT, 1%  $\beta$ -mercaptoethanol, 5 ng/ml bovine serum albumin, 1.5 units/ml aprotinin, and 1  $\mu$ g poly(dI.dC) (Pharmacia). DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide gel at 4 °C in  $0.5 \times$  TBE buffer ( $1 \times$  TBE = 0.09 M Tris-borate and 0.002 M EDTA). Gels were fixed in 50% ethanol and 10% acetic acid for 30 min, followed by drying. The radioactive gels were analyzed by PhosphorImager (Molecular Dynamics). As a probe, we used the double-strand oligonucleotides of PPRE (GTTCGACAGGGGACCAGGACAaAGGTTCACGTTCCGGAGTTCGAC), obtained from the rat acyl-CoA oxidase promoter [17].

## 2.8. Western blot analysis

Before treatment with Wy-14643 or 1,25-(OH) $_2$ D $_3$ , we maintained the cells for 1 week in Dulbecco's modified Eagle's medium containing 10% charcoal-stripped fetal calf serum, and then either  $10^{-5}$  M WY-14643 or  $10^{-7}$  M 1,25-(OH) $_2$ D $_3$  was added to HaCaT keratinocytes cultured on 100-mm dishes at subconfluency. At the indicated times after the treatment, keratinocytes were washed twice with ice-cold phosphate-buffered saline and extracted with the extraction buffer (1% NP-40, 1  $\mu$ g/ml aprotinin, and 400 mM NaCl in 20 mM HEPES/pH 7.2). Protein concentration was determined using Bradford reagent (Bio-Rad). Twenty or forty

microgram per lane of cell lysate protein from keratinocytes were loaded onto 10% polyacrylamide gel, and transferred to an Immobilon-P membrane (Millipore). The membrane was soaked overnight in 5% skim milk in phosphate-buffered saline/0.05% Tween-20 at 4 °C and then incubated with anti-TR2 monoclonal IgM [8,13] at a 1:800 dilution in 5% skim milk in phosphate-buffered saline/0.05% Tween-20 for 2 h at room temperature. After being washed for three 10 min intervals with phosphate-buffered saline/0.05% Tween-20, the membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgM (PharMingen) at a 1:1000 dilution for 1 h at room temperature. Detection was performed with Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad). The expression amounts of TR2 were quantified by collage image analysis software (Fotodyne).

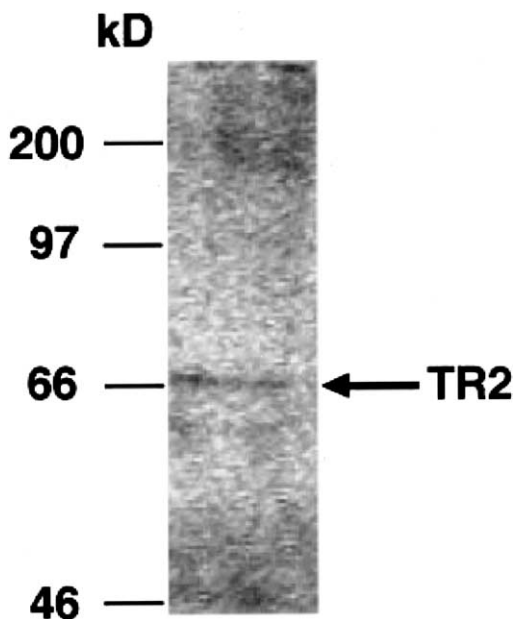
## 3. Results

### 3.1. TR2 protein is expressed in HaCaT keratinocytes

Western blot analysis of human HaCaT keratinocytes cell lysates were performed to examine TR2 expression. The TR2 protein with a molecular weight of 67 kDa was detected with monoclonal anti-TR2 antibody (Fig. 1). With the isotype control IgM, this band was not detected (data not shown). The specificity of this monoclonal antibody has been confirmed previously [8,20].

### 3.2. TR2 suppresses RA-induced transactivation in HaCaT keratinocytes

Our group previously reported that TR2 suppresses RA-induced transactivation in CV1 cells [9]. We have reported that TR4 can suppress RA-, but not vitamin D-induced transactivation in human HaCaT keratinocytes [21]. Here we further examined the potential effect of increased TR2 expression on RA- and vitamin D-induced transactivation in HaCaT keratinocytes by a CAT reporter gene assay. To assay the RA- and vitamin D-induced transactivation, we used the pRAR $\beta$ -CAT reporter plasmid that contains the synthetic RA response element sequence (GGTTCaccgaaAGTTCA) obtained from the promoter region of RAR $\beta$  [15], and the P450cc24-CAT reporter plasmid that contains the 5'-flanking region (-2200 to +188) of the rat vitamin D $_3$  24-hydroxylase gene [16]. To analyze ligand-induced transactivation of CAT reporter plasmid via endogenous cognate receptors

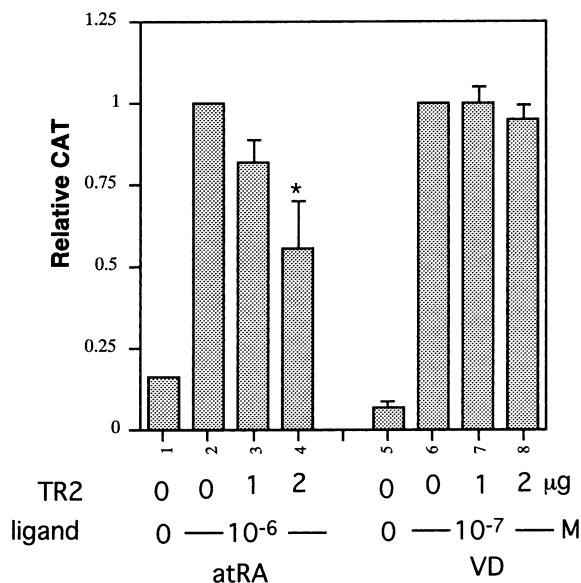


**Fig. 1** TR2 protein is expressed in HaCaT keratinocytes. Twenty microgram of cell lysate from subconfluent HaCaT keratinocytes were subjected to SDS-PAGE and Western blotting, followed by immunoblotting with the monoclonal antibody against TR2. Bars on the left margin represent the molecular mass marker in kilodaltons (kDa).

in HaCaT keratinocytes, exogenous RARs and vitamin D receptor were not transfected. We observed a 6.3-fold induction by  $10^{-6}$  M *all-trans* RA in the absence of TR2 expression vector (Fig. 2, lane 1 vs. 2). Co-transfection of 1 and 2  $\mu$ g of pCMV-TR2 decreased this CAT activity by 18.3 and 44.7%, respectively (Fig. 2, lane 3 and 4). Although we detected a 15.9-fold induction by  $10^{-7}$  M of 1,25-(OH) $_2$ D $_3$  without exogenous TR2 (Fig. 2, lane 5 or 6), co-transfection of 1 or 2  $\mu$ g of pCMV-TR2 did not have a significant effect on CAT activity (Fig. 2, lanes 7 and 8). Co-transfection of pCMV-TR2 had no effect on the basal (ligand-independent) CAT activity (data not shown). These results suggested that TR2 could suppress RA-, but not vitamin D-induced transactivation in human HaCaT keratinocytes.

### 3.3. TR2 can bind specifically to PPRE

Recently, it has been reported that PPAR $\alpha$  ligands can enhance keratinocyte differentiation [12]. Therefore, it will be of interest to examine whether there is any cross-talk between these two receptor signaling pathways. First, we examined whether TR2 can bind to PPRE using an electrophoretic mobility shift assay. The in vitro translated TR2 was incubated with  $^{32}$ P-labeled

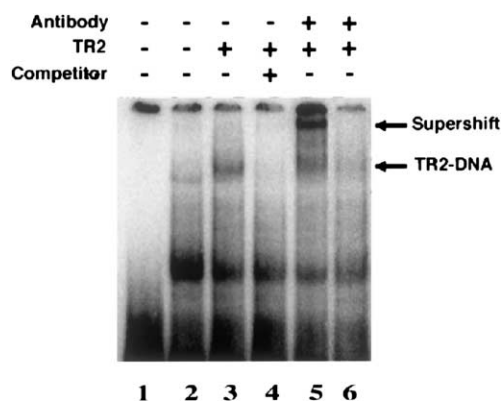


**Fig. 2** TR2 can suppress RA-induced pRAR $\beta$ -CAT, but not vitamin D-induced P450cc24-CAT reporter activity. The reporter plasmids (4  $\mu$ g of pRAR $\beta$ -CAT and P450cc24-CAT) were transfected into HaCaT keratinocytes in the presence of the indicated amount of pCMV-TR2 plasmid. The cells were treated with  $10^{-6}$  M *all-trans* RA,  $10^{-7}$  M 1,25-(OH) $_2$ D $_3$ , or ethanol only, as indicated. CAT activities were determined and expressed as relative activity of each reporter in the presence of *all-trans* RA or 1,25-(OH) $_2$ D $_3$ , but in the absence of pCMV-TR2 plasmid. Error bars represent the mean  $\pm$  S.D. from three independent experiments. \*Significant difference from the control (lane 2),  $P < 0.01$ .

PPRE (GTCGACAGGGGACCAGGACAaAGGTCACGTTCCGGAGTCCGAC), obtained from the rat acyl-CoA oxidase promoter. A specific TR2-PPRE complex was shown in the presence of TR2 but absent in the mock translated control (Fig. 3, lane 3 vs. lane 2). This TR2-PPRE complex could be abolished with a 100-fold molar excess of the unlabeled PPRE (Fig. 3, lane 4). Moreover, this retarded complex was supershifted in the presence of the rabbit anti-TR2 serum (Fig. 3, lane 5), but could not be seen with the pre-immune rabbit serum (Fig. 3, lane 6). Together, these data clearly demonstrated that TR2 could bind specifically to PPRE.

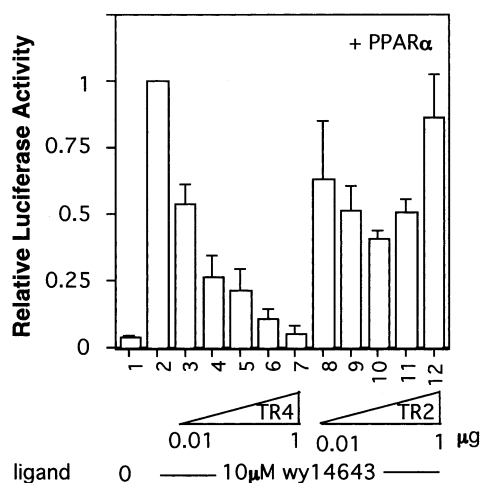
### 3.4. TR2 and TR4 differentially suppress PPAR transactivation in HaCaT keratinocytes

Next, we examined the effects of TR2 and TR4 on PPAR $\alpha$  transactivation by luciferase assays. Since our preliminary studies indicated that PPRE $_3$ -luciferase activity without exogenous PPAR $\alpha$  transfection is too low to detect, the PPAR $\alpha$  expression vector pSG5-PPAR $\alpha$  was transfected into HaCaT keratinocytes. As shown in Fig. 4, both TR2 and



**Fig. 3** TR2 can specifically bind to PPRE. In vitro translated TR2 (1  $\mu$ l) was incubated with 0.1 ng  $^{32}$ P-labeled PPRE oligomer and analyzed by an electrophoretic mobility shift assay. Lane 1, labeled probe alone; lane 2, the probe and the mock translated control; lane 3, the probe and the in vitro translated TR2 protein; lane 4, the probe, the TR2 protein, and a 100-fold molar excess of PPRE; lane 5, the probe, the TR2 protein, and anti-TR2 serum; lane 6, the probe, the TR2 protein, and pre-immune serum.

TR4 can suppress Wy-14643 (a PPAR $\alpha$ -specific ligand)-induced PPAR $\alpha$  transactivation. The suppression effects by TR4 and TR2, however, are



**Fig. 4** TR2 and TR4 differentially suppress PPAR transactivation in HaCaT keratinocytes. The reporter plasmids (4  $\mu$ g of PPRE $_3$ -tk-LUC) were transfected into HaCaT keratinocytes in the presence of various amounts (lanes 1 and 2, 0  $\mu$ g; lanes 3 and 8, 0.01  $\mu$ g; lanes 4 and 9, 0.25  $\mu$ g; lanes 5 and 10, 0.5  $\mu$ g; lanes 6 and 11, 0.75  $\mu$ g; lanes 7 and 12, 1  $\mu$ g) of pCMV-TR2 or pCMX-TR4. The cells were treated with  $10^{-5}$  M WY-14643 or ethanol only, as indicated. Luciferase activities were determined and expressed as relative activity of the reporter in the presence of Wy-14643, but in the absence of pCMV-TR2 or pCMX-TR4. Error bars represent mean  $\pm$  S.D. from three independent experiments. \*Significant difference from the control (lane 2),  $P < 0.01$ .

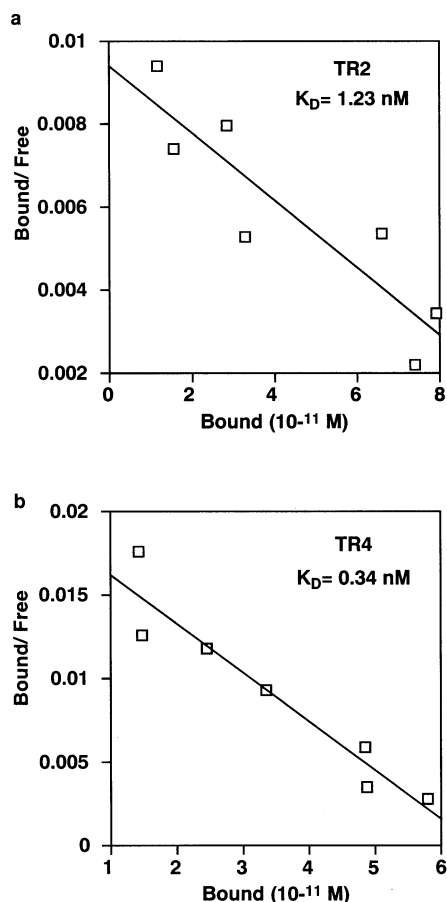
quite different: only TR4 can repress PPAR $\alpha$  signal pathway in a dose dependant manner reaching a maximal of 95% suppression (Fig. 4, lanes 3–7). In contrast, TR2 can first repress PPAR $\alpha$  to 59.8% (Fig. 4, lane 9 and 10) and then, surprisingly, the suppression gradually disappears with the addition of more TR2. (Fig. 4, lanes 8–12). Together, these data clearly suggest that the PPAR $\alpha$  signaling pathway can be differentially regulated by TR2 and TR4, two very closely related members in the orphan nuclear receptor superfamily.

### 3.5. Differential binding affinity of TR2 and TR4 toward PPRE

To clarify the potential mechanism for the differential regulation of PPAR $\alpha$  signal pathway by TR2 and TR4, the Kds (equilibrium dissociation constants) for TR2 and TR4 binding to PPRE were calculated using Scatchard analysis. As shown in Fig. 5a and b, these Kds for TR2 and TR4 were 1.23 and 0.34 nM, respectively, indicating that the affinity for TR4 is higher than that for TR2. This result may provide one explanation of how TR2 and TR4 can differentially repress the PPAR $\alpha$  signal pathways. The Kd values calculated here were consistent with the range of Kd values for nuclear receptors and their hormone response elements. Together, these data clearly suggest that the PPAR $\alpha$  signal pathway can be differentially regulated by the closely related TR2 and TR4 in HaCaT keratinocytes.

### 3.6. Wy-14643 upregulates the expression level of TR4 protein but downregulates that of TR2 protein in HaCaT keratinocytes

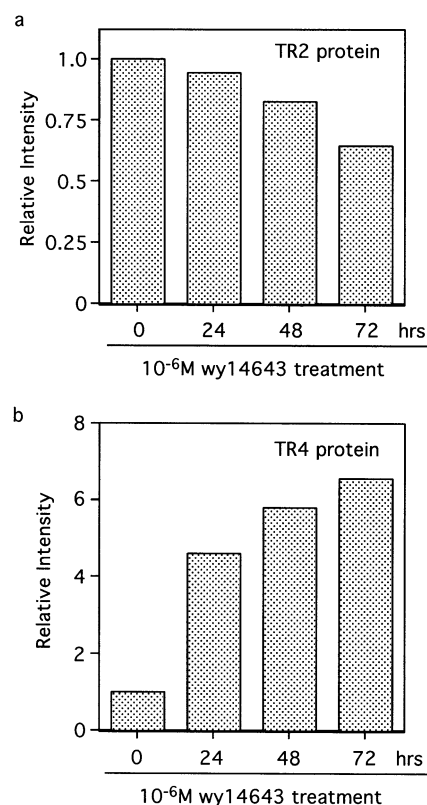
In order to investigate whether TR2 and TR4 can act as negative feedback regulators, we tested the effect of WY-14643 on the expression level of TR2 and TR4 protein using Western blot analysis. Following treatment with  $10^{-5}$  M Wy-14643. TR2 decreased to 82 and 66% at 48 and 72 h, respectively (Fig. 6a). In contrast, TR4 protein expression increased approximately 4.58-, 5.80-, and 6.56-fold at 24, 48, and 72 h, respectively, suggesting that TR4 can act as a strong negative feedback modulator for PPAR $\alpha$  in human HaCaT keratinocytes (Fig. 6b). These results indicate that TR2 and TR4 are differentially regulated by Wy-14643, and this may have different implications for modulation of PPAR $\alpha$  activity. As a control,  $10^{-7}$  M 1,25-(OH) $_2$ D $_3$  had no effect on TR2 and TR4 protein expression levels (data not shown).



**Fig. 5** The affinity of TR2 for binding to PPRE is weaker than that of TR4. Scatchard analysis of the binding of the <sup>32</sup>P-labeled probe (PPRE) to in vitro translated orphan receptors TR2 (a) and TR4 (b). Band shifts were developed and quantified by IMAGEQUANT software. To quantify the total probe in the reaction, a known amount of the probe was spotted onto a filter paper and then quantified by IMAGEQUANT software.

#### 4. Discussion

Comparison of amino acid sequences between TR2 and TR4 [2–4] shows that these two orphan nuclear receptors share very high (64%) sequence homology. Their DNA-binding domains (DBD) have 81% homology with 100% homology in the P-box, a very important domain within DBD for recognition of the hormone response elements in the target genes. This high sequence homology between TR2 and TR4 suggests that they may have very similar biochemical and physiological functions. Indeed, our previous reports indicated that TR2 and TR4 have very similar effects on the following signal pathways: (1) thyroid hormones [5,6], (2) ciliary neurotropic factor receptor [7,8], (3) RA [9,10], (4) simian virus 40 gene [11,22], and (5) erythropoietin gene expression [20]. In this study, we clearly



**Fig. 6** Wy-14643 upregulates the expression level of TR4 protein and decreases the expression level of TR2 protein. At 0, 24, 48, and 72 h after the addition of 10<sup>-5</sup> M Wy-14643, HaCaT keratinocytes were harvested. Forty microgram of cell lysates were subjected to Western blot analysis for TR2 (a) or TR4 (b). Each upper graph is the quantification of each band in the lower immunoblot.

demonstrated that TR2 and TR4 have distinct functions through differential and bi-directional regulation in the PPAR $\alpha$  signal pathway. This is the first report to indicate that closely related members of the nuclear receptor superfamily, such as TR2 and TR4, can have distinct functional characteristics.

To explore the potential mechanisms causing the differential regulation of TR2/TR4 and PPAR $\alpha$ , the affinities of TR2 and TR4 binding to PPRE-DNA were measured. Scatchard analysis showed that the DNA-binding affinity of TR4 is higher than that of TR2, which may allow TR4 to act as a more sensitive modulator for PPAR $\alpha$ . Alternatively, the putative cofactors for TR2 and TR4 that interact differentially with TR2 and TR4 may explain this difference. Recent studies in the nuclear receptor area have suggested that cofactors for nuclear receptors can greatly modulate the receptor activity. Further characterization of TR4 cofactors may provide more information about how TR2 and TR4 can differentially regulate their target genes.

In conclusion, our findings here are the first evidence that closely related members in a nuclear receptor subfamily, such as TR2 and TR4, can have distinct functions through differential and bi-directional regulation with PPAR $\alpha$  signal pathway. This discovery may provide a very interesting model to study the molecular mechanism and biochemical function of TR2 and TR4 in human keratinocytes.

## Acknowledgements

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