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Induction of apolipoprotein E expression by TR4 orphan nuclear receptor via 5′ proximal promoter region

Eungseok Kim*, Zhiming Yang, Ning-Chun Liu, Chawnshang Chang

George Whipple Lab for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, and The Cancer Center, University of Rochester Medical Center, Rochester, NY 14642, USA

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Abstract

While other plasma lipoproteins are exclusively expressed in liver and intestine, apoliprotein E (apoE) is ubiquitously synthesized in many tissues. To understand the molecular mechanism of non-tissue-specific apoE expression, we tested the testicular orphan receptor 4 (TR4) effect on apoE expression in different cell lines, such as HepG2, COS-1, and H1299 cells. Gel shift assay and 5′ promoter activity analyses identified one distinct hormone response element (TR4RE-DR0-apoE at −303 to −292 bp) that binds to TR4 and results in full induction of apoE gene transcription. TR4 also forms a complex with Sp1 to synergistically induce apoE expression via a region containing the TR4RE-DR0-apoE and the Sp1 binding site (−169 to −140 bp). Induction of apoE expression by TR4 was also confirmed at the mRNA and protein levels in H1299 cells. Together, our data demonstrate that TR4 can enhance apoE gene expression via binding to TR4RE-DR0 in apoE 5′ promoter and this TR4 binding is essential for synergistic interaction with another transcription factor, Sp1.

Keywords: TR4; apoE; DR0; Sp1

Members of the nuclear receptor superfamily are transcription factors that regulate gene expression through the binding to specific DNA sequences known as hormone response elements (HREs). These nuclear receptors include receptors for steroids, thyroid, vitamin D3, retinoids, and a large number of orphan receptors with no known ligands [1].

The human and rat testicular orphan receptor 4 (TR4) cDNAs were isolated from testis, prostate, and hypothalamus cDNA libraries that showed a high degree of sequence homology (65%) with the testicular orphan receptor 2 (TR2) [2]. TR4 shows ubiquitous expression in different tissues [2,3] and can regulate the expression of target genes through binding to AGGTCA direct repeats (DRs) in its target genes [4–8]. TR4 also participates in diverse signaling pathways such as androgen, retinoid, thyroid hormone, vitamin D3, and the ciliary neurotrophic factor (CNTF) through either binding to its target DNA or protein–protein interaction [5–7,9].

Apolipoprotein E (apoE) is mainly synthesized in liver, yet widely expressed in different tissues including brain, adrenal, ovary, testis, and kidney [10,11]. ApoE is an important component of very low density lipoprotein and chylomicrons, and serves as a ligand for the receptor-mediated hepatic uptake of these lipoproteins [12]. ApoE is also involved in pathogenesis of atherosclerosis through the modulation of cholesterol efflux from macrophages [13]. Among three isoforms, apoE4 has been linked to atherosclerosis [14] and Alzheimer's disease [15], and apoE3 has been suggested to have a neuroprotective role in excitotoxin-induced neuronal damage [16]. Furthermore, apoE can interact with CNTF and may have some roles in CNTF-mediated survival-promoting activity in cultured hippocampal neurons [17]. The pivotal roles of apoE in these

* Corresponding author. Fax: +1 585 756 4133.
E-mail address: eungseok_kim@urmc.rochester.edu (E. Kim).

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pathways are well established in apoE knockout mouse studies [18–20]. ApoE expression has been shown to be modulated by tissue-specific enhancers in different tissues. In transgenic mouse studies, apoE expression in liver is promoted by hepatic control regions (HCRs) [21] and recently, TR4 was shown to regulate expression of the apoE/C-I/C-II gene cluster via TR4 response element located within HCR-1 [4]. In contrast, liver X receptor (LXR)/retinoid X receptor heterodimers have been shown to regulate apoE expression in macrophages and adipocytes by direct binding to DR4 in another tissue-specific enhancer, multienhancer domain, of the human apoE/C-I/C-I′/C-IV/C-II gene cluster [22,23]. However, the mechanism of how the expression of apoE, a ubiquitously expressing apolipoprotein, is regulated in many tissues remains unclear. With wide overlapping of tissue distribution between apoE and TR4, it will be interesting to see if there is any potential effect of TR4 in non-tissue-specific apoE 5′ proximal promoter region.

Here, we demonstrate that TR4 can significantly induce apoE gene expression not only through the tissue specific enhancer, HCR, but also through the apoE 5′ proximal promoter region via TR4RE-DR0. We also showed that TR4 can cooperate with Sp1 to synergistically induce apoE gene expression via TR4RE-DR0 and Sp1 binding site (−169 to −140 bp). These results suggest important roles for the TR4 signaling pathway in lipid and lipoprotein metabolism.

Materials and methods

Plasmids. pCMX-TR4 and pGL-apoE have been described [4] and pCMV-hSp1 was from Guntram Suske (Philipps-Universität, Marburg). Various deletions of apoE 5′ promoter were produced by PCR from pGL-apoE and subcloned into pGL3-luciferase (Promega) or pGL3-TK-luciferase in which we cloned thymidine kinase (TK) promoter (−32 to +48 bp) into pGL3-luciferase. Synthesized DR0 oligonucleotides were subcloned into pGL3-TK-luciferase to create pGL-TK-TR4RE-DR0-apoE.

Cell culture and transfection. HepG2, COS-1, and H1299 cells were maintained in Dulbecco's Minimum Essential Medium containing 10% fetal calf serum. Transfections were performed using SuperFect (Qiagen).

Gel shift assay. Gel shift assays were performed as described [4] with the use of in vitro translated proteins and ^{32}P-labeled oligonucleotide probes. The following oligonucleotides were used in the gel shift assay: TR4RE-DR0-apoE (5′-GAAAGGACAGGGTCA-GGA-3′) and mutated TR4RE-DR0-apoE (5′-GAAACCACAGCCTCAGGA-3′). For the antibody supershift assays, anti-TR4 monoclonal antibody was added to the reaction. DNA–protein complexes were resolved on a 5% native gel and analyzed by Storm PhosphorImager (Amersham).

Co-immunoprecipitation. TR4 and Sp1 were in vitro translated in the presence of [^{35}S]Met using TNT Coupled Reticulocyte Lysate System (Promega). Twenty microliters of [^{35}S]Met TR4 protein was incubated with an equal volume of either [^{35}S]Met Sp1 protein or mock-translated lysate for 1 h at room temperature. Anti-Sp1 antibody (Santa Cruz) was added to the reaction and immunoprecipitation was performed as described [4]. Immunoprecipitated proteins were separated by SDS–PAGE (8% gel) and visualized by Storm PhosphorImager (Amersham).

Chromatin immunoprecipitation (ChIP). Cells were fixed by formaldehyde (final conc. 1%) and then lysed in lysis buffer (5 mM Pipes/pH 8.0, 85 mM KCl, and 0.5% NP-40 with Roche protease inhibitor mixture). The nuclei were collected and lysed in nuclear lysis buffer (50 mM Tris/pH 8.1, 10 mM EDTA, and 1% SDS with Roche protease inhibitor mixture), and incubated on ice for 10 min. The samples were sonicated to an average length of ~600 bp. The chromatin solution was precleared with protein A–Sepharose (Santa Cruz) for 30 min at 4 °C and immunoprecipitations (IPs) were performed overnight, at 4 °C, with 1 μg of normal mouse IgG, anti-TR4 antibody, or anti-Sp1 antibody. After immunoprecipitation, immunocomplexes were precipitated by the addition of 45 μl protein A–Sepharose. Precipitates were washed sequentially for 3–5 min each in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl/pH 8.1, and 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl/pH 8.1, and 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris–HCl/pH 8.1), and twice in 1× TE buffer. After removal of cross-link by extraction using 1% SDS and 0.1 M $NaHCO_3$ and incubation at 65 °C, DNA fragments were purified with a QIAquick Spin Kit (Qiagen, CA). For PCR detection of TR4 responsive region of apoE promoter, the primers were: 5′-CCTCCCATCCCACTTCTG-3′ (forward), 5′-ACCTCGCCCAGTAATACAG-3′ (reverse).

Western blot analysis. Media samples from H1299 cells transfected with a TR4 expression vector or an empty vector were tested for newly synthesized apoE by Western blot analysis. Samples were separated by 10% SDS–PAGE. After electrophoresis, proteins were transferred from the gel to Immobilin P transfer membrane (Millipore) and apoE was resolved using anti-apoE polyclonal antibody (Chemicon) and alkaline phosphatase-conjugated secondary antibody (Bio-Rad).

Analysis of gene expression by real time quantitative RT-PCR. Total RNA was extracted from H1299 cells transfected with a TR4 expression vector or an empty vector using Trizol reagent (Invitrogen). Quantitative real-time PCR was carried out using iCycler iQ Real-Time RT-PCR Detection system (Bio-Rad) with conditions and reagents as recommended by the manufacturer. Each sample was analyzed in triplicate. Sequence-specific RT-PCR primers for apoE and 18S cDNAs were used as follows: 18S: primers, 5′-TGCACCACCAACTGCTTAGC-3′ and 5′-CAGTCTTCTGAGTGGCAGTGATG-3′, and apoE: primers, 5′-GCGTTGCTGGTCACATTCC-3′ and 5′-GCTCTGCCACTCGGTCTG-3′. The 18S was used as endogenous control for normalization of cDNA amounts.

Results

TR4 induces apoE gene 5′ promoter activity

Recently, we reported that in HepG2, human hepatoma cells, TR4 induces transcriptional activity of apoE gene in reporter gene assays not only through HCR-1, liver-specific enhancer, but also 5′ proximal promoter, although TR4-induced 5′ promoter activity is lower than the basal activity of HCR-1 [4]. This suggests that TR4 may regulate non-tissue-specific apoE expression via the 5′ promoter, even though this TR4 effect may not contribute significantly to hepatic apoE expression. To test this hypothesis, we determined whether TR4 could induce non-tissue-specific apoE expression by induction of apoE 5′ promoter activity in HepG2, COS-1, and H1299 cells using the reporter gene consist-

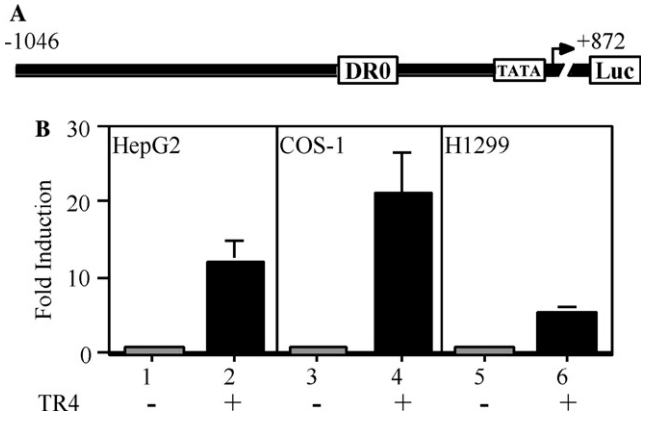

Fig. 1. Induction of apoE 5′ promoter activity by TR4. (A) Schematic representation of human apoE 5′ promoter luciferase construct (pGL-apoE). The nucleotides are numbered relative to the transcription initiation site and putative TR4 response element (DR0) is shown. (B) TR4 activates apoE 5′ promoter activity in HepG2, COS-1, and H1299 cells. Cells were transfected with 500 ng pGL-apoE in the presence or absence of 200 ng pCMX-TR4. The relative luciferase activities were determined by comparison with luciferase activity of the empty vector (set as 1-fold) and the results are expressed as means ± SD of three separate experiments.

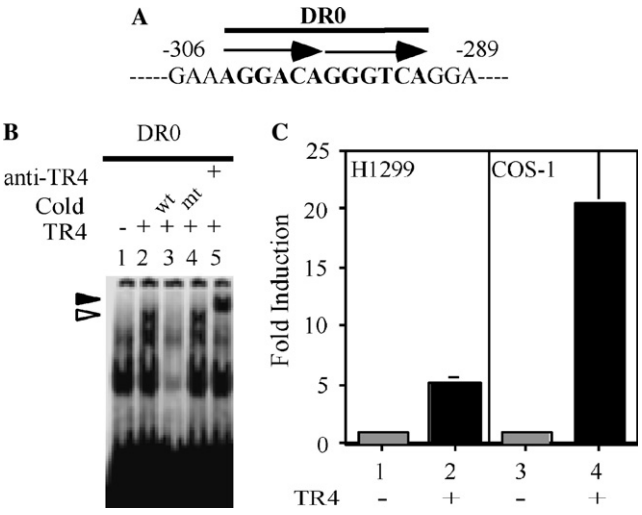

Fig. 2. TR4 activates apoE 5′ promoter activity through specific binding to TR4RE-DR0-apoE. (A) ApoE 5′ promoter region containing putative TR4 response element, DR0. AGGTCA-like sequences are indicated by arrows. (B) Gel shift assay was performed using ^{32}P-labeled TR4RE-DR0-apoE probe with in vitro translated TR4. We added 100 molar excesses of unlabeled oligonucleotides (wt) or mutated oligonucleotides (mt) as a competitor. For supershift assay, anti-TR4 antibody was added as indicated. Retarded complex and supershifted band are indicated by open and closed arrow heads, respectively. (C) Functional analysis of the TR4RE-DR0-apoE regulated by TR4 co-transfected 500 ng pGL-TK-TR4RE-DR0-apoE with 200 ng pCMX-TR4 into H1299 or COS-1 cells. Relative luciferase activities are expressed as fold-induction compared to transfection of the empty vector (set as 1-fold).

ing of −1046 to +872 bp promoter region (Fig. 1A). As shown in Fig. 1B, TR4 can induce apoE 5′ promoter activity 5- to 26-fold in various cells, suggesting that TR4 can induce apoE expression via induction of apoE 5′ promoter activity.

Identification of TR4-HRE (TR4RE-DR0-apoE) in the apoE 5′ promoter region

Sequence analysis of apoE 5′ promoter region between −1046 and +872 bp reveals one potential TR4-HRE, DR0, (AGGACAGGGTCA), located at −303 to −292 bp (Fig. 2A). Gel shift assay was applied to test if this DR0 HRE (named TR4RE-DR0-apoE) could bind specifically to TR4. As shown in Fig. 2B, [^{32}P]-TR4RE-DR0-apoE forms a specific band after incubation with in vitro translated TR4, but not mock-translated control (lane 2, open arrowhead vs. lane 1). This band could be abolished by addition of 100 molar excesses of unlabeled TR4RE-DR0-apoE (lane 3). In contrast, this band remained intact after adding 100 molar excess of unlabeled mutated TR4RE-DR0-apoE (lane 4). As expected, this band could be supershifted with anti-TR4 antibody (lane 5, closed arrowhead). Together, data from gel shift assay demonstrate that TR4 can bind specifically to TR4RE-DR0-apoE in the 5′ promoter region.

To further test if TR4RE-DR0-apoE is a real HRE that could mediate the TR4-induced apoE 5′ promoter activity, we linked TR4RE-DR0-apoE to a luciferase reporter and assayed the TR4 effect in H1299 and COS-1 cells. As shown in Fig. 2C, addition of TR4 can induce the luciferase activity (lanes 2 and 4), suggesting that TR4RE-DR0-apoE is a real TR4-HRE that mediates TR4 induction of apoE 5′ promoter activity.

TR4 cooperates with Sp1 to synergistically induce apoE 5′ promoter activity

An earlier study showed that the region between −360 and −15 bp was needed for full activity of apoE 5′ promoter. Several transcription factors including Sp1 have been reported to regulate apoE gene by binding to this region [24], which contains three Sp1 binding sites (−184 to −173 bp, −169 to −140 bp, and −59 to −45 bp). Other reports also suggest that Sp1 can induce its target gene expression via cooperation with some members of the nuclear receptor superfamily, such as estrogen receptor and androgen receptor [25,26]. Therefore, we were interested in determining if TR4 could also cooperate with Sp1 to induce apoE 5′ promoter activity. We first tested whether Sp1 has an effect on TR4-mediated apoE 5′ promoter activity. As shown in Fig. 3A, we were not able to see the Sp1 effect on apoE 5′ promoter activity although we could see TR4-mediated apoE 5′ promoter activity (lanes 2 and 3). The difference between our result and previous report of Sp1 effect on apoE promoter activity may be due to the different lengths

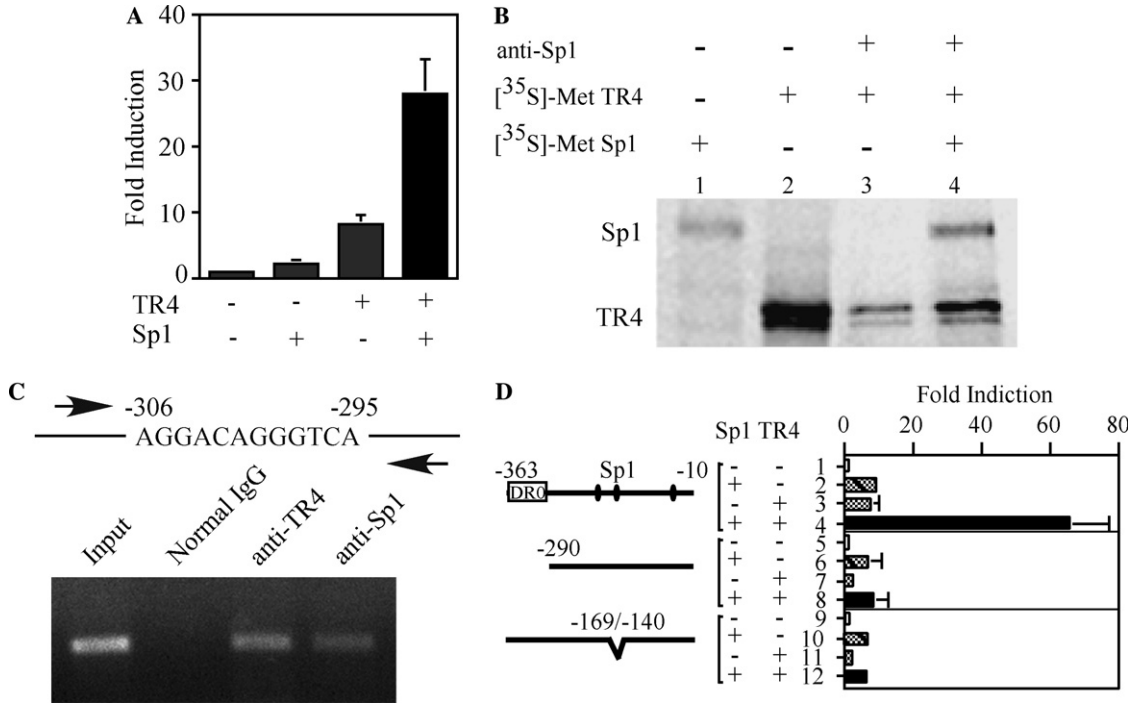

Fig. 3. Synergistic induction of apoE 5′ promoter activity through the interaction between TR4 and Sp1. (A) Effect of Sp1 on TR4-mediated transcriptional activity in apoE 5′ promoter. We co-transfected 100 ng pCMX-TR4 or pCMV-hSp1 with 300 ng reporter (pGL-apoE) linked with full-length apoE 5′ promoter into HepG2 cells as indicated. Relative luciferase activities are expressed as fold-induction compared to transfection of the empty vector (set as 1-fold). (B) Co-immunoprecipitation with anti-Sp1 antibody was performed with in vitro translated [^{35}S]Met TR4 protein in the presence or absence of in vitro translated [^{35}S]Met Sp1 protein. (C) Analysis of TR4 and Sp1 interactions with the apoE promoter by ChIP assay. After immunoprecipitation of cross-linked complexes from HepG2 cells transfected with TR4 and Sp1 expression vectors, the chromatin was analyzed by PCR as described under Materials and methods. Single PCR products were obtained for antibodies as indicated. (D) Deletion analysis of apoE promoter. We co-transfected 100 ng pCMX-TR4 or pCMV-hSp1 with 300 ng luciferase reporters linked with serial deletions of apoE 5′ promoter into HepG2 cells as indicated. The nucleotides of promoter regions linked to luciferase reporter are indicated, and TR4RE-DR0-apoE (DR0) and Sp1 binding sites (Sp1) are shown. Relative luciferase activities are expressed as a fold induction compared to transfection of the empty vector (set as 1-fold) in each reporter gene.

of the promoter we used. However, when Sp1 and TR4 were co-transfected (lane 4), we found synergistic enhancement up to 3.5-fold of TR4-mediated transcriptional activity of apoE 5′ promoter.

We then performed co-immunoprecipitation assay using [^{35}S]Met in vitro translated TR4 and Sp1 proteins to determine whether this synergistic cooperation is through physical interaction between TR4 and Sp1. As shown in Fig. 3B, anti-Sp1 antibody strongly co-immunoprecipitated TR4 together with Sp1 (lane 4). In contrast, TR4 was barely immunoprecipitated by anti-Sp1 antibody when TR4 was added alone (lane 3). Considering the higher sensitivity of radioactivity, this marginal effect of anti-Sp1 antibody on TR4 immunoprecipitation may be non-specific binding of TR4 to anti-Sp1 antibody. Interaction of TR4 with Sp1 within apoE 5′ promoter was also investigated in HepG2 cells co-transfected with TR4 and Sp1 using a chromatin immunoprecipitation (ChIP) assay. After formaldehyde cross-linking and shearing of chromatin by sonication, protein–DNA complexes were immunoprecipitated with normal serum IgG, anti-TR4, or anti-Sp1 antibody. PCR was performed with the primers directed to −410 to −201 bp region encompassing the putative TR4 binding site, TR4RE-DR0-apoE. As expected, TR4 was recruited to this apoE promoter region via the TR4RE-DR0-apoE (Fig. 3C). No PCR product was observed using DNA immunoprecipitated with normal serum IgG. We were also able to see that anti-Sp1 antibody could immunoprecipitate this promoter region although the amplified PCR product band was weaker than that of anti-TR4 antibody, suggesting that Sp1 may be associated with this promoter region via TR4/Sp1 interaction.

To further determine whether DNA binding of these transcription factors is necessary for synergistic cooperation, we first constructed a reporter using −363 to −15 bp, known to be important for Sp1 function on apoE 5′ promoter activity, and then performed reporter gene assays using reporters fused to various deletion mutant promoters. As demonstrated in Fig. 3D, TR4 was able to significantly induce activity of this reporter gene containing intact TR4RE-DR0-apoE (lane 3). In contrast, TR4 failed to activate transcription of the

reporter gene, when this TR4RE was deleted (lane 7). Studies with serial deletions of apoE 5′ promoter demonstrate that TR4RE-DR0-apoE as well as Sp1 binding site (−169 to −140 bp) is important to mediate this TR4/Sp1 synergistic induction of apoE expression (Fig. 3D, lane 4 vs. 8 and 12). Surprisingly, if the Sp1 binding site (−169 to −140 bp) was deleted, we found that the TR4 induction effect was also destroyed (Fig. 3D, lane 3 vs. 11), suggesting that Sp1 in the −169 bp to −140 bp region may stabilize the TR4 binding to the TR4RE-DR0-apoE. Together, results from Figs. 3A–D demonstrate that TR4 can interact and cooperate with Sp1 to synergistically induce apoE 5′ promoter activity. This synergistic effect between TR4 and Sp1 may go through DNA-binding in the neighboring sequence of apoE 5′ promoter.

TR4 induces apoE gene expression

ApoE protein is known as a secreting protein and is present in plasma. Therefore, to study the effect of TR4 on apoE expression, we then compared the apoE protein level in media from H1299 cells transfected with either a TR4 expression vector or an empty vector as a control. After recovery overnight, cells were subjected to serum-free media for 3 h, and newly synthesized and secreted apoE levels were determined by immunoblotting the media using anti-apoE antibody. As shown in Fig. 4A, addition of TR4 to H1299 cells can significantly increase apoE protein level in the media. This

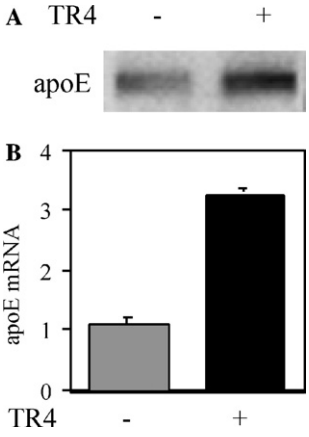

Fig. 4. TR4 induces apoE protein and mRNA levels. (A) H1299 cells were transfected with a TR4 expression vector or a control vector (pCMX-TR4 or pCMX) for 12 h. Media were replaced with fresh media containing 10% fetal calf serum, and cells were grown for another 12 h. Cells were then subjected to serum-free media for 3 h and the media were harvested. Proteins synthesized and secreted to the media were separated by 10% SDS–PAGE and immunoblotted by anti-apoE antibody. (B) H1299 cells were transfected with pCMX-TR4 or pCMX as described above. Total RNA from H1299 cells was prepared as described under Materials and methods. Relative mRNA expression of apoE gene was quantified by RT-PCR and the results are expressed as means ± SD of three individual experiments.

induction was further confirmed at the mRNA level using quantitative real-time RT-PCR assays. As shown in Fig. 4B, the level of apoE mRNA was 3.25 ± 0.13-fold higher in H1299 cells transfected with TR4 expression vector relative to empty vector-transfected H1299 cells.

Discussion

ApoE is widely expressed in many tissues including brain, adrenal, lung, ovary, testis, and kidney, and apoE expression has been shown to be modulated by tissue-specific enhancers in different tissues [4,22,23]. However, regulation of apoE expression only through tissue-specific enhancers cannot explain ubiquitous expression of apoE in many tissues. In the present study, we demonstrated that TR4 modulated non-tissue-specific transcriptional activity of apoE gene by recognition of TR4RE in apoE 5′ promoter and through functional synergy with Sp1.

ApoE promoter has a DR0 located on −303 to −292 bp, and TR4 can bind to this site. Recently, we showed that TR4 can induce hepatic apoE expression via liver-specific enhancer, HCR-1, and this suggests that TR4 may regulate tissue-specific or/and non-tissue-specific transcriptional activities of apoE gene in different tissues upon the availability of tissue-specific transcription factors or coregulators. In HepG2 cells, we were also able to see that TR4-mediated induction of apoE 5′ promoter activity although it did not contribute much to hepatic apoE expression. Another nuclear receptor, LXR, has been shown to regulate tissue-specific apoE expression in non-hepatic cells such as macrophages and adipocytes. However, there is no report on the role of LXR in hepatic apoE expression, even though it is highly expressed in liver. This suggests that LXR may also need other tissue-specific factors to regulate apoE expression in these non-hepatic cells.

The region between −360 and −15 bp has been well known to be important for transcriptional activity of apoE promoter, and several transcription factors including Sp1 have been known to bind to this region. One TR4RE, DR0, is also located within this region of the apoE 5′ promoter. Although DR0 has a low affinity for TR4, reporter gene assays showed that TR4 highly induces the apoE promoter activity of this region (Fig. 3D). Early studies suggested that Sp1 could interact with various nuclear receptors, including AR and ER to modulate their target gene expression. The interaction between Sp1 and nuclear receptors not only enhances transcriptional activity of the nuclear receptor itself, but also promotes nuclear receptor modulation of target gene expression indirectly through interaction with Sp1 on Sp1 binding sites in the 5′ promoter regions [26,27]. We found that the −169 to −140 bp region in the 5′

promoter region is very important for the TR4/Sp1 complex to maintain both the Sp1 and TR4 effect on the induction of apoE expression.

This suggests that TR4 and Sp1 form a complex with each other upon binding to their respective sites in apoE 5′ promoter and this complex may recruit coregulators and general transcription factors to form a preinitiation complex for non-tissue-specific apoE transcription. An alternative hypothesis is that other transcription factors and coregulators which bind to other regions of the apoE promoter might strengthen the interaction between TR4 and Sp1 in this region of apoE promoter. To understand the role of other transcription factors and coregulators on TR4/Sp1 synergy in non-tissue-specific apoE promoter activity, further study will be needed.

ApoE is synthesized in many tissues and TR4 is also expressed in these tissues including the liver. This wide overlapping of tissue distribution in TR4 and apoE expression further strengthens the regulation of apoE expression by TR4 in vivo.

In summary, our studies demonstrate that TR4 may be able to modulate apoE expression via multiple mechanisms and these findings may provide us some potential physiological implications of TR4 in lipid metabolism.

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