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Thyroid hormones upregulate apolipoprotein E gene expression in astrocytes

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ABSTRACT

Apolipoprotein E (apoE), a protein mainly involved in lipid metabolism, is associated with several neurodegenerative disorders including Alzheimer's disease. Despite numerous attempts to elucidate apoE gene regulation in the brain, the exact mechanism is still uncovered. The mechanism of apoE gene regulation in the brain involves the proximal promoter and multienhancers ME.1 and ME.2, which evolved by gene duplication. Herein we questioned whether thyroid hormones and their nuclear receptors have a role in apoE gene regulation in astrocytes. Our data showed that thyroid hormones increase apoE gene expression in HTB14 astrocytes in a dose-dependent manner. This effect can be intermediated by the thyroid receptor β (TRβ) which is expressed in these cells. In the presence of triiodothyronine (T3) and 9-cis retinoic acid, in astrocytes transfected to overexpress TRβ and retinoid X receptor α (RXRα), apoE promoter was indirectly activated through the interaction with ME.2. To determine the location of TRβ/RXRα binding site on ME.2, we performed DNA pull down assays and found that TRβ/RXRα complex bound to the region 341–488 of ME.2. This result was confirmed by transient transfection experiments in which a series of 5′- and 3′-deletion mutants of ME.2 were used. These data support the existence of a biologically active TRβ binding site starting at 409 in ME.2. In conclusion, our data revealed that ligand-activated TRβ/RXRα heterodimers bind with high efficiency on tissue-specific distal regulatory element ME.2 and thus modulate apoE gene expression in the brain.

1. Introduction

Apolipoprotein E (apoE) is a glycoprotein with a major involvement in lipid metabolism, mediating lipoprotein clearance from the plasma. Besides its role as a lipid carrier from astrocytes to neurons via cell surface receptors, apoE plays an important role in neurobiology and neurodegenerative disorders including Alzheimer's disease (AD) [1]. Astrocytes are the principal apoE suppliers in the brain [2,3].

Studies regarding apoE gene expression revealed a complex network of interactions between regulatory elements, triggering cell-specific expression. Human apoE gene is located in apoE/apoCI/apoCI/apoCIV/apoCII gene cluster on chromosome 19 [4]. Two distal duplicated sequences, multienhancer 1 and 2 (ME.1 and ME.2) identified by Grehan et al. [5] control apoE expression in astrocytes, since in their absence, apoE is not expressed in these cells. A series of transcription factors and nuclear receptors regulate apoE, upon binding on its proximal promoter or on other regulatory elements [6]. Among the nuclear receptors, RXRα and LXRα were reported to modulate apoE gene expression [7].

The thyroid hormones can regulate gene expression through their interaction with the thyroid hormone receptor (TR). TR is a member of the nuclear receptors family with important role in modulating key functions, such as cell growth and differentiation [8], development, lipid and carbohydrate metabolism [9,10], and cardiovascular function [11]. In the brain, TRs are involved in

Abbreviations: AD, Alzheimer's disease; apoE, apolipoprotein E; LXR, Liver X Receptor; ME.2, Multienhancer 2; RA, 9-*cis*-Retinoic Acid; RXR, Retinoid X Receptor (RXR); T3, L-3,5,3′-triiodothyronine; Thyroid Hormone Receptor β, TRβ; TO, TO-901317.

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fundamental processes such as neural development, differentiation, synaptogenesis, myelination, signaling and cell migration [12]. Moreover, a connection between AD risk and TR gene polymorphisms has been reported [13]. After ligand binding, TRs regulate cell processes acting on gene expression, usually as heterodimers with RXR. Triiodothyronine (T3) shows a great affinity for the TR/RXR complex, while 9-*cis*-Retinoic Acid (RA) induces the release of co-repressors without conformational change of the complex formed by the two partners [14]. In addition, it was demonstrated that TRs cross-talk with other nuclear receptors such as PPAR [15], LXR [16], RAR [17], GR [18]. Several isoforms of TR were identified so far, among which TRα1, TRβ1 and TRβ2 contain DNA- and ligand (T3)- binding domains [19]. TRα1 and TRβ1 are nearly ubiquitously expressed, while TRβ2 is expressed in the developing brain, hypothalamus, and anterior pituitary gland, as reviewed in Ref. [9].

We hypothesize that thyroid hormones and their receptors have a role in apolipoprotein regulation in the brain. Herein we present data demonstrating that thyroid hormones upregulate apoE gene in astrocytes by a mechanism involving long range interactions between apoE promoter and ME.2, which binds ligand-activated TR. These data are particularly relevant for the prevention or future treatments of apoE-related neurodegenerative diseases.

2. Materials and methods

2.1. Reagents

DMEM and fetal bovine serum (FBS) were from Invitrogen (Life Technologies), Luciferase Assay System was from Promega (Madison, WI). The primers were from Invitrogen (Life Technologies), except biotinylated RV3 primer and primers for TRβ, which were from Microsynth AG (Switzerland). Anti-TRβ monoclonal antibodies (J51, sc-737), anti-LXRα/β rabbit polyclonal antibodies (H-144, sc-13068) and anti-β-actin monoclonal antibodies (C4; sc-47778) were from Santa Cruz Technology (Santa Cruz, CA). Anti-human apoE (A299) rabbit antibodies were from Immuno-Biological Laboratories Co., LTD. (Aramachi, Takasaki-Shi, Gunma, Japan). T3, 9-cis-Retinoic Acid, and TO901317 were from Sigma. Streptavidin-Dynabeads M-280 were from Thermo Fisher Scientific. InnuPREP RNA Mini kit was from Analytik Jena (Jena, Germany), high-capacity cDNA Reverse Transcription kit was from Applied Biosystems, and SuperSignal West Pico substrate from Pierce Biotechnology, Rockford, USA. Charcoal-stripped serum was from SIGMA.

2.2. Plasmid construction

The TRβ expression vector was obtained by excising the cDNA encoding THRβ gene from the pCR-BluntI-TOPO vector (clone MHS4426-98361206 from Open Biosystem-Thermo Scientific) with HindIII and XbaI and ligating it into pCMV-SPORT6 expression vector. Human apoE promoter alone (−500apoE) or together with multienhancer 2 (ME.2/-500apoE) was cloned in pGL3 basic vector in front of luciferase reporter gene, as previously described [20]. ME.2 (19–619 ME.2) and its deletion mutants (341–619 ME.2, 509–619 ME.2, 19–488 ME.2, and 19–298 ME2) were amplified with the primers described in Ref. [20], except the primer F509: 5′-GGGGTACCCAGAGGGTGAATAAGAGCAG which was used as forward for the amplification of the fragment 509–619. The fragments were cloned in pGL3 vector containing a minimal promoter described in Ref. [21].

2.3. Cell culture and transfection

HTB14, HEK 293 and HepG2 cells (from ATCC) were grown in DMEM supplemented with 10% FBS. Cells were transiently transfected as described in Ref. [20], then FBS was replaced with charcoal stripped serum and the transfected cells were incubated for 24 h with ligands for TRβ, RXRα, LXRα (75 nM T3, 1 μM 9-*cis*-Retinoic Acid, and 10 μM TO901317, respectively). The activity of the luciferase reporter gene was determined with a FB12 Luminometer (Berthold), as previously described [22].

2.4. RT-PCR

Total RNA from HTB14 cells was extracted using InnuPREP RNA Mini kit. cDNA was obtained from 1 μg of RNA using High-Capacity cDNA Reverse Transcription kit. The primers used for apoE and GAPDH were described in Ref. [20]. For human TRβ, the primers were: 5′-TCCTTCTGGTTGGCTGTCCTGCGTG (forward) and 5′-TGGTCTGGATGAGATGTGGCGACG (reverse).

2.5. Western blot

Ligand-treated and control HTB14 cells were washed in PBS, solubilized, subjected to SDS-PAGE and then transferred onto nitrocellulose membranes (Bio-Rad). After blocking, the membranes were incubated overnight (4 °C) with the primary antibodies (anti-apoE, TRβ and β-actin), followed by incubation with HRP-conjugated secondary antibodies. The bands were detected with SuperSignal West Pico substrate with LAS-4000 Chemiluminescent Image Reader (FUJIFILM Europe GmbH, Germany).

2.6. DNA pull-down assay (DNAP)

ME.2 (19–619) or its fragments (19–141, 19–488, 341–488, 267–619) were amplified by PCR using biotinylated RV3 forward primer, reverse primers and corresponding plasmids containing ME.2 fragments, as described in Ref. [20]. Whole cell extract from HEK 293 cells overexpressing TRβ and RXRα was obtained as previously described [22] and incubated with the biotinylated DNA immobilized on Streptavidin-Dynabeads for 16 h at 4 °C in binding buffer described in Ref. [20]. The complexes were washed 10 times with binding buffer (4 °C), and subjected to Western blot using anti-TRβ and anti-RXRα antibodies. The bands were detected as above.

2.7. Statistical analysis of the data

All experiments described above were run in triplicates and repeated at least three times. Fold-changes were calculated as ratios of the averages between treated and control samples. Statistical analysis was performed using one-way analysis variance between groups with OriginPro 7.5. All values were expressed as means ± S.E; $p < 0.05$ was considered statistically significant.

3. Results

3.1. T3 and 9-cis-Retinoic Acid significantly enhance apoE expression in astrocytes

To test the capacity of T3 to modulate apoE gene expression in astrocytes, HTB14 cells were exposed to increasing concentrations of hormone (from 7.5 nM to 750 nM), in the presence of 1 μM RA. In addition, we tested apoE expression in astrocytes treated with 1 μM RA alone or in the presence of 10 μM TO, to compare the effect of TR/RXR heterodimers with the activity of RXR homodimers or LXR/

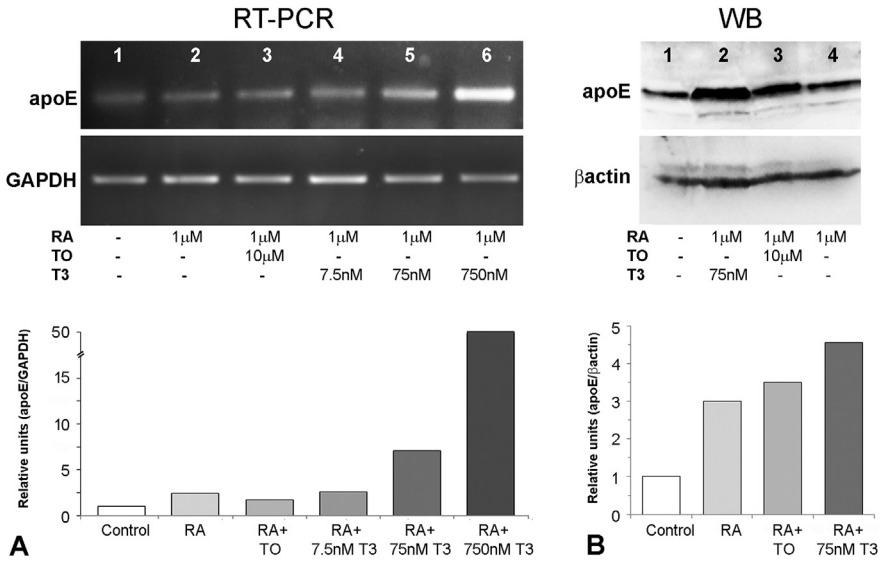

Fig. 1. In astrocytes, ligand-activated RXR and its heterodimerization partners, TR and LXR, modulate apoE expression at gene and protein level. (A) Astrocytes incubated with RA alone or in combination with 1 μM TO (LXR ligand) or 7.5 nM T3 exhibit a ~2.5 fold increase in apoE mRNA level; T3 modulates apoE gene expression in a dose-dependent manner in HTB14 cells, as detected by RT-PCR, up to a ~50 fold increase for 750 nM T3. **(B)** ApoE protein level increases more in cells treated with T3 in the presence of RA (4.5 times) than in cells exposed to RA alone or in combination with TO, as revealed by Western blot. GAPDH and β-actin were used as internal control for RT-PCR and Western blot, respectively. Representative experiments are illustrated.

RXR heterodimers. Total RNA from the treated cells was extracted and analyzed by RT-PCR. The results obtained showed that apoE expression in cells exposed to RA or with the combination of RA and TO increased ~2.4 fold compared with control cells (Fig. 1A, lanes 1–3). The combination between T3 and RA increased apoE gene expression in a dose-dependent manner. Thus, increasing T3 concentrations from 7.5 nM to 75 nM and further to 750 nM induced an increase in apoE expression by 2.5, 7 and 50 times, respectively (Fig. 1A, lanes 4–6).

ApoE protein expression in astrocytes was assessed by Western blot using cells treated with 75 nM T3 in the presence of 1 μM RA; in addition, the effect of cell treatment with 10 μM TO in the presence of RA, or only with RA for 24 h was also tested. β-actin expression was assessed for normalization. Data showed that apoE protein level increased ~4.5 times upon exposure to T3 and RA (Fig. 1B, lane 2) as compared with HTB14 control cells (Fig. 1B, lane 1). Cell treatment with the LXRα/RXRα ligands (TO and RA) augmented apoE expression in astrocytes, but the induction was smaller than that of TR ligands, namely 3.5 times for RA with TO and ~3 times for RA alone (Fig. 1, lanes 3, 4). Similar results were obtained for another human tumor brain cell line, HTB13 (data not shown).

Next, we determined TRβ expression in HTB14 cells and found that HTB14 cells express TRβ at both mRNA and protein levels (Fig. 2A and B).

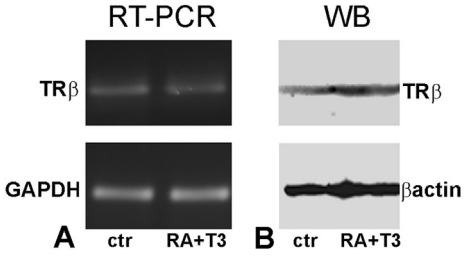

Fig. 2. HTB14 astrocytes express TRβ. TRβ expression in cultured HTB14 cells untreated or exposed to T3 and RA was assessed by RT-PCR (**A**) or Western Blot (**B**). TRβ is detected at mRNA and protein level in both untreated and treated astrocytes. GAPDH and β-actin were used as internal controls for RT-PCR and Western blot, respectively.

3.2. *In astrocytes, ligand-activated TRβ/RXRα heterodimer binds on ME.2 and induces apoE promoter activity via long range interactions*

We further tested whether TRβ/RXRα overexpression in the presence of T3 and RA modulates the activity of apoE proximal promoter directly or indirectly via multienhancer 2. Astrocytes were transiently co-transfected with plasmids containing apoE proximal promoter (−500apoE) or constructs containing ME.2 in front of apoE proximal promoter in pGL3 basic vector (ME.2/-500apoE), in the presence of expression vectors for RXRα alone or together with TRβ or LXRα. Eighteen hours after transfection, the corresponding ligands were added: RA for RXRα, T3 and RA for TRβ/RXRα, and T0 and RA for LXRα/RXRα. Overexpression of ligand-activated TRβ/RXRα heterodimers had no effect on apoE promoter (Fig. 3A, RA + T3 column), but produced a significant upregulatory effect on apoE promoter linked to ME.2 (Fig. 3B, column RA + T3). Overexpression of RXRα alone or with LXRα in the presence of the corresponding ligands raised ~9 times apoE promoter activity only in the presence of ME2, but not in the absence of ME.2 (Fig. 3B and A, respectively). The same effects were detected in HTB11 neuroblastoma cells (not shown). By contrast, similar experiments in HepG2 revealed no activation by T3 on apoE promoter in the presence or absence of ME.2 (Fig. 3C and D). These data revealed that T3 modulation on apoE promoter is cell-type specific, due to the selective action of ME.2.

3.3. *TRβ/RXRα binding site is located on ME.2*

To identify the location of TRβ/RXRα binding site on ME.2 fragment, we analyzed the binding of TRβ and RXRα on full-length ME.2 or its deletion fragments 19–141, 19–488, 341–488, 267–619. For this, we performed DNAP experiments using biotinylated full-length or ME.2 fragments and whole cell extracts obtained from HEK 293 cells overexpressing TRβ and RXRα. The TRβ and RXRα bound to 5′-biotinylated ME.2 were precipitated with Streptavidin-

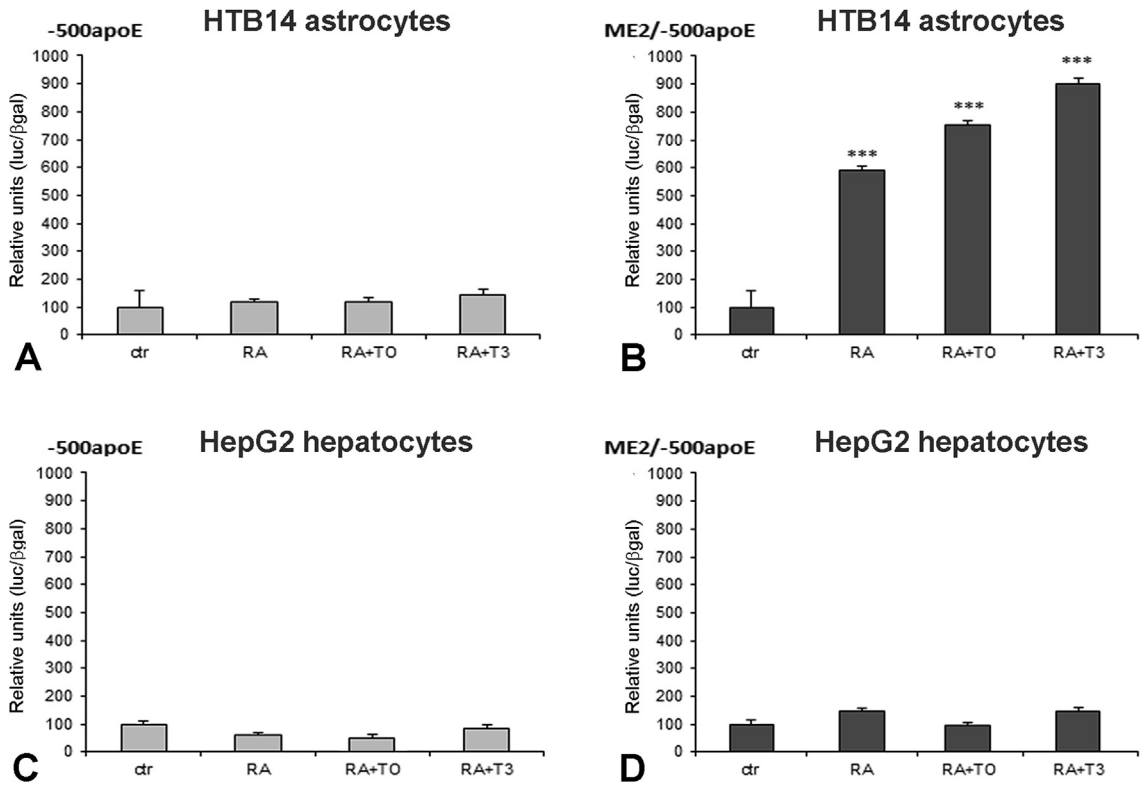

Fig. 3. Ligand-activated TRβ/RXRα heterodimers act on the distal regulatory element ME.2 and transactivate apoE promoter in astrocytes. Transient transfections were performed using plasmids containing apoE promoter cloned in pGL3 vector (−500apoE, **A, C**) and plasmids containing ME.2 in front of apoE promoter (ME.2/-500apoE, **B, D**). The cells were co-transfected with expression vectors for RXRα, TRβ/RXRα and LXRα/RXRα and treated with the corresponding ligands (RA, RA + T3 and RA + TO, respectively). In the absence of ME2, apoE promoter activity is not modulated by the nuclear receptors tested either in astrocytes or in hepatocytes (**A, C**, respectively). In the presence of ME.2, in astrocytes (**B**) but not in hepatocytes (**D**) apoE promoter activity is significantly increased ($p < 0.001$) by RXRα, LXRα/RXRα and TRβ/RXRα, the latter exhibiting the highest activity.

Dynabeads and identified by Western blot. Ligand-activated TRβ and RXRα bound to ME.2 and to 19–488, 341–488, 267–619 fragments of ME.2, but not to 19–141 fragment (Fig. 4A, right). As positive control, whole cell extracts prepared from TRβ- and RXRα-overexpressing HEK 293 cells were used (Fig. 4A, right, lane NE). No binding was detected for random DNA (Fig. 4A, lane Rnd). These results indicated that a TRβ/RXRα binding site is located in the 341–488 region of ME.2, as schematically illustrated in Fig. 4A, left.

To confirm the presence of TRβ/RXRα binding site on ME.2, series of 5′ and 3′ deletion mutants of ME.2 placed in front of a minimal promoter were tested for their capacity to be activated by RA and T3 treatment in cells overexpressing TRβ and RXRα. The results showed that the activities of 5′-deletion mutant 341-619ME.2 and 3′-deletion mutant 19-488ME.2 are increased by TRβ/RXRα (Fig. 4B). By contrast, shorter mutants such as 509-619ME.2 and 19-298ME.2 are not activated by TRβ/RXRα (Fig. 4B). These data corroborate with DNAP results and confirm the localization of TRβ/RXRα binding site within the region 341–488 of ME.2.

4. Discussion

In the brain, astrocytes represent the main apoE supplier, since through the brain–blood barrier only a small fraction of high density lipoproteins are transported from the blood [23]. Consequently, apoE biosynthesis and secretion by astrocytes are vital to warrant the proper apoE level in the brain. The regulatory mechanisms of apoE biosynthesis in the brain are not entirely elucidated, but data indicate that the hormonal control has a significant role. Among the hormones, estrogens were shown to increase apoE expression in mouse brain [24,25]. Since astrocytes play an essential role in the metabolism of thyroid hormones in the brain, being involved in thyroxine transport from the blood and its conversion to the active form 3,5,3′-triiodothyronine (T3), these cells are considered the main source of T3 for the central nervous system [26]. Thus, we focused on T3 role in apoE gene regulation in astrocytes. We show here that treatment with T3 in the presence of RA induces in astrocytes an increase in apoE expression in a dose-dependent manner (Fig. 1). Similar results were obtained on HTB13 astrocytoma cell line (data not shown). Since RXRα can homodimerize or heterodimerize with LXRα, we treated HTB14 cells with RA alone, or in combination with LXRα ligand, TO and followed-up apoE expression. In the astrocytes treated with TO and RA, apoE expression was positively modulated, but to a lower extent than that obtained with T3 and RA. Our data revealed that this positive regulation of apoE expression at mRNA level is also found at the protein level.

Herein we report for the first time the influence of thyroid hormones on apoE expression. Data from the literature refers to the upregulatory effect of the thyroid hormones on various other apolipoproteins such as apolipoprotein CII [27], apolipoprotein M [28] and apolipoprotein A5 [29]. Moreover, a decreased level of apolipoprotein B was reported after administration in patients of a thyromimetic compound (eprotirome) [30]. The modulatory action of T3 on apolipoprotein expression has consequences in lipid metabolism, and thus the thyroid hormones became a therapeutic target for hyperlipidemia. Due to the involvement of thyroid hormones in various processes, thyromimetic compounds that act in a tissue-specific manner were developed. For example, eprotirome [30] and indane derivatives [31] are potent liver-specific thyromimetics, lowering plasma cholesterol and avoiding heart toxicity and

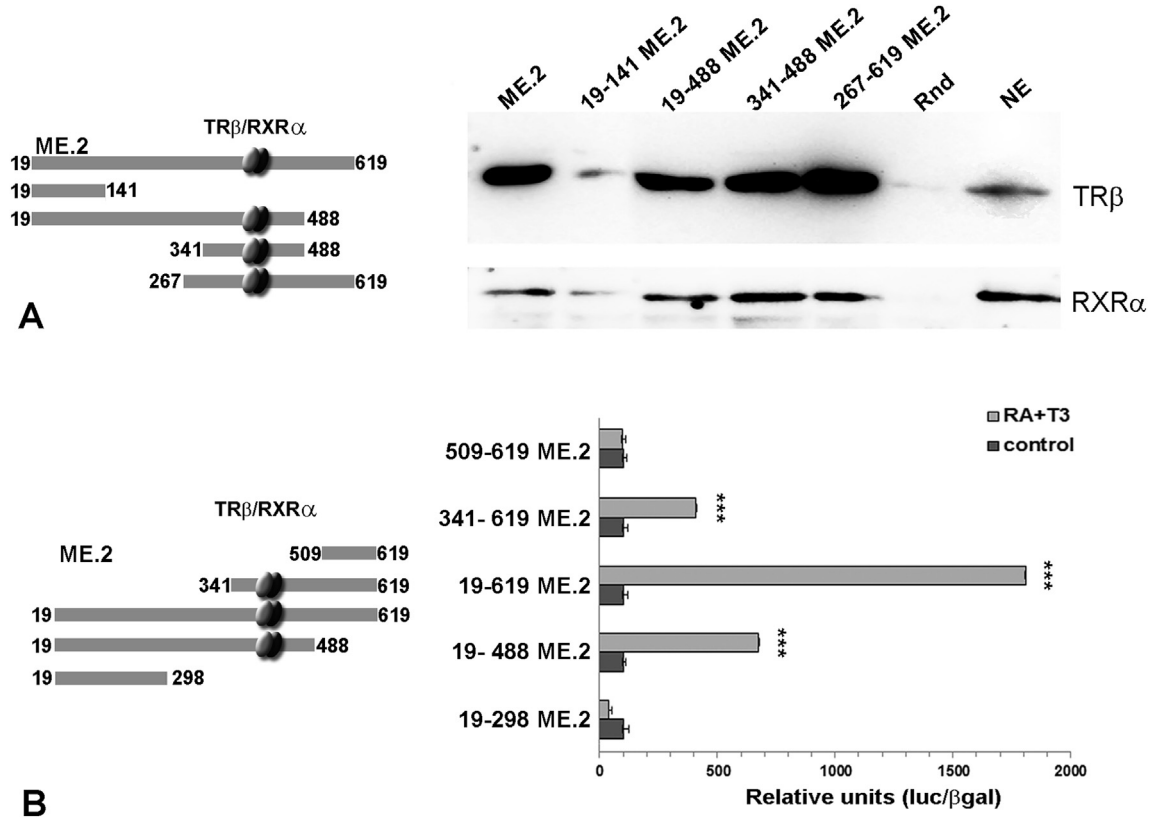

Fig. 4. Localization of TRβ/RXRα binding site on ME.2. (A) *In vitro* TRβ and RXRα binding to the full-length and some ME.2 fragments was determined by DNA pull down assay. Briefly, biotinylated DNA incubated with nuclear extract from cells transfected to overexpress TRβ and RXRα was precipitated with Streptavidin-Dynabeads; bound TRβ and RXRα were identified by Western Blot. TRβ and RXRα bind to the entire ME.2 (19–619) and its fragments 19–488, 341–488, 267–619, but not to the 19–141 fragment, depending on the presence or absence of the binding site (schematically illustrated on the left side). No band is present in the negative control (random DNA, Rnd); the positive control is represented by Western blot of the nuclear extract of the TRβ and RXRα transfected cells (NE). **(B)** Ligand-activated TRβ/RXRα heterodimers transactivate ME.2 or its deletion mutants containing TRβ/RXRα binding site. Transient transfections were performed using ME.2 deletion mutants cloned in front of a minimal promoter in pGL3 basic vector. The activity of luciferase reporter gene was enhanced by the ligand-activated TRβ/RXRα when ME.2 fragments contain the putative TRβ/RXRα binding site (341–619, 19–488); mutants 509–619 and 19–298, from which the TRβ/RXRα was deleted, could not be stimulated by TRβ/RXR overexpression.

other side effects. Our data showed that TRβ are expressed in HTB14, data in agreement with the reported TRβ2 expression in astrocytes [32].

In the brain, macrophages, and adipose cells, apoE expression is controlled by two distal multienhancers, ME.1 and ME.2, localized 3.3 kb and respectively 15 kb downstream of apoE gene, which evolved through gene duplication [5,33]. Despite their 95% homology, ME.2 seems to be more active in apoE induction in the brains of transgenic mice [5], and thus we focused on ME.2 in the current work. Our results showing enhanced apoE expression by the ligands of RXRα or LXRα/RXRα in astrocytes are similar with the data obtained on macrophages [34].

To investigate the mechanisms underlying TRβ action on apoE gene regulation, first we tested the ability of the ligand-activated TRβ/RXRα to activate apoE promoter alone or in the presence of ME.2. Our transient transfection experiments in astrocytes using plasmids containing apoE proximal promoter revealed that only in the presence of ME.2, apoE promoter is transactivated by RXRα/RXRα, TRβ/RXRα and LXRα/RXRα. In HepG2 cells, where ME.2 is not active, RXRα overexpression alone or in the presence of TRβ or LXRα did not enhance apoE promoter activity regardless ME.2 presence (Fig. 3C and D). Thus, we focused on ME.2 in the search for a TRβ binding site. Using TFBIND Search software [35] we identified a putative TRβ binding site starting at 409 in ME.2, which might play a role in the regulation of apoE gene expression. Thereafter we used two alternative techniques to assess the biological activity of TRβ binding site. First, we tested the binding *in vitro* by DNAP, in which the TRβ/RXRα bound to ME.2 or its deletion fragments were identified by Western Blot.

Then, we questioned if the activated complex TRβ/RXRα overexpressed in HTB14 cells is able to increase the activity of ME.2 deletion mutants and found that TRβ/RXRα binding site on ME.2 identified by *in silico* analysis is biologically active. Interestingly, this region is overlapping with a sequence that was previously identified as a LXR binding site [7]. This may be explained by the similitude of the binding sites of these two nuclear receptors. Moreover, a competition for the promoter activation between TRβ and LXRα, up-regulating the important Seladin-1 gene involved in AD, was reported [36].

In conclusion, our results show that in astrocytes (i) ligand activation of TRβ/RXRα complex is implicated in apoE gene regulation; (ii) the interaction between hormone-responsive TRβ and RXRα, which bind to ME.2 and not to apoE promoter; (iii) the binding site of TRβ/RXRα heterodimer is located at 409 bp in ME.2. Taken together, our data indicates that the regulation of apoE by thyroid hormones is cell-specific and is done via long range interactions in astrocytes. The new findings regarding the T3 signaling pathway in the brain emphasize the role of thyroid hormone on apoE gene regulation, and may contribute to the development of novel therapies for the treatment of apoE-related neurodegenerative diseases.

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