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Thyroid hormone receptor mediates human *MDR1* gene expression—Identification of the response region essential for gene expression

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ABSTRACT

P-glycoprotein, encoded by the *MDR1* gene, is a drug efflux transporter that is expressed in various tissues and plays an important role in the absorption and elimination of many drugs and xenobiotics. Induction of the *MDR1* gene affects drug disposition and the efficacy of drug treatment. In this study, we demonstrated that the thyroid hormone receptor (TR) induces *MDR1* gene expression in a thyroid hormone (TH)-dependent manner. The 5′-upstream region of the human *MDR1* gene was examined for the presence of TH-responsive elements. Luciferase-reporter gene assays revealed that the TH response region is located between −7.9 and −7.8 kb upstream from the transcription start site of *MDR1*. The region contains two TH response clusters, one of which includes a direct repeat with a three-nucleotide spacer (DR3) and a four-nucleotide spacer DR4(I), and the other of which includes two DR4s (II and III). Mutation analyses indicated that every direct repeat has a unique contribution to the TH response. In particular, DR4(I) was shown to be the most important element. Chromatin immunoprecipitation assays revealed that TR and retinoid X receptor (RXR) bind to the TH response region, and gel mobility shift assays confirmed that one molecule of TR/RXR heterodimer binds to each of the clusters in this region, with preferential binding to the upstream one. We furthermore demonstrated that two molecules of TR/RXR could bind simultaneously to the TH response region. The order of binding affinity to the direct repeats was DR4(I) > DR4(II) > DR4(III) ≈ DR3. Our results indicate that these two closely spaced TR/RXR-binding clusters are both required for the maximal induction of *MDR1* gene expression mediated by TR.

The *MDR1* (*ABCB1*) gene plays an important role in pharmacokinetics through the expression of efflux pump P-glycoprotein (P-gp)[1], which has a broad substrate specificity and tissue-specific distribution. P-gp is expressed at high levels on the apical/luminal surface of barrier (blood–brain barrier, intestine, placenta, blood–testis, and blood–ovarian barriers) and excretory (liver, kidney, adrenal gland) tissues, suggesting that P-gp can limit the cellular uptake of drugs into the brain, testis, fetus, and enterocytes and can eliminate drugs into the bile, urine, and intestinal lumen [1,2]. The expression of *MDR1* is induced by a variety of drugs, affecting pharmacokinetics and leading to a decrease in the systemic exposure of drugs that serve as P-gp substrates. The inducers, inhibitors, and tissue distribution patterns of P-gp and CYP3A4 overlap extensively, with the latter being the most abundantly expressed human cytochrome P450 that contributes to the metabolism of a wide spectrum of pharmaceutical drugs and xenobiotics [3,4]. Consequently, the expressions of *MDR1* and *CYP3A4* were expected to have similar induction mechanisms. In fact, the induction of both *MDR1* and *CYP3A4* was found to be mediated through transcriptional activation by the binding of nuclear receptors, such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR), to distal enhancer elements located approximately 8 kb upstream from the transcription start sites of *MDR1* and *CYP3A4* [5–8].

In vivo drug transport can be radically changed by a variety of pathophysiological states, including thyroid dysfunction. For example, in patients with hyperthyroidism who are receiving digoxin—a well-known P-gp substrate—the renal clearance of digoxin is higher and the plasma concentration of digoxin is lower than after normalization of thyroid function [9]. P-gp expressed in the kidneys and intestine has a substantial effect on the urinary and plasma concentration of orally administered P-gp substrates. For example, the intestinal P-gp content is correlated with the AUC after the oral administration of digoxin [10]. Therefore, the alterations in the pharmacokinetics of digoxin in patients with hyperthyroidism can be explained by the induction of P-gp; in other words, the expression level of *MDR1* may increase in patients with hyperthyroidism which is characterized by higher levels of thyroid hormone. Siegmund et al. reported that the expression of intestinal

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[1] *Abbreviations used:* P-gp, P-glycoprotein; TH, thyroid hormone; TR, thyroid hormone receptor; DR, direct repeat; PXR, pregnane X receptor; CAR, constitutive androstane receptor; RXR, retinoid X receptor; TRE, thyroid hormone response element; UpC, upstream cluster; DwC, downstream cluster; CYP, cytochrome P450; MDR1, multidrug resistance 1; FITC, fluorescein isothiocyanate; ChIP, chromatin immunoprecipitation.

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P-gp increased after the oral administration of thyroxine (T4) in duodenal biopsy samples obtained from healthy human volunteers [11]. Mitin et al. demonstrated that T4 induced MDR1 mRNA and P-gp in human colon carcinoma cell lines [12]. Although these results indicate that thyroid hormone (TH) is involved in the induction of *MDR1* gene expression, there have been no direct evidence that the thyroid hormone receptor (TR) is involved in the induction.

THs, 3,5,3′-triiodothyronine (T3) and T4, modulate gene expression by interacting with thyroid hormone receptors (TRs), α and β, which are members of the nuclear receptor superfamily [13]. T3 is the principal active and main ligand of TR and is derived by the deiodination of T4. TRα and TRβ, encoded by two distinct genes—*THRA* (*NR1A1*) and *THRB* (*NR1A2*) [13,14], bind T3 and, with a lesser affinity, T4, and mediate TH-regulated gene expression. TR binds to TH-responsive element (TRE), typically as a heterodimer with retinoid X receptor (RXR). TREs consist of a direct repeat, an everted repeat, or an inverted repeat of the consensus hexamer (half-site) sequence of (G/A)GGT(C/G)A separated by a spacer that is several nucleotides long; in particular, DR4 (a direct repeat separated by four nucleotides) is a preferential site for TR binding [15].

Overt thyroid dysfunction is common in the general population [16]. Hypothyroidism is a common disorder affecting many people, especially those over the age of 60 years [17,18]. Patients with hypothyroidism are usually treated with TH replacement therapy. Since TH is widely prescribed and influences the induction of P-gp, which potentially affects pharmacokinetics, the role of TH in the mechanism of *MDR1* expression may be a worthwhile topic of study. In this study, we investigated the molecular mechanism of *MDR1* induction by TH using intestinal epithelial cell lines. We demonstrated that TR/RXR heterodimers bind to the TREs located −7.9 to −7.8 kb upstream from the transcription start site of *MDR1*, which is essential for the induction of *MDR1* by TH.

Materials and methods

Cell cultures and hormone treatment

Human intestinal epithelial cell lines LS180 and Caco-2 were obtained from American Type Culture Collection (Manassas, VA) and were routinely cultured in DME low-glucose medium D6046 (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS), 1× nonessential amino acids (Life Technology), and penicillin (100 IU/ml)/streptomycin (100 μg/ml) (Life Technology) at 37 °C in a humidified atmosphere of 5% CO_2. To study the thyroid hormone actions, the medium was substituted with phenol red-free DME medium (Gibco, Invitrogen) supplemented with 10% dextran-coated charcoal-treated fetal bovine serum (Hyclone Inc.) for 24 h prior to treatment with vehicle (2 mM NaOH), L-thyroxine (T4, Sigma), 3,3′,5,-triiodo-L-thyronine (T3, Sigma), or 3,3′,5′,-triiodo-L-thyronine (rT3, Sigma). T4, T3, and rT3 stock solutions were prepared in 0.2 M NaOH and were added to the medium at a final concentration of 2 mM NaOH. For the time course experiments, LS180 cells were treated with T4 (5 μM) for 4–24 h followed by RNA preparation. For the concentration–response studies, LS180 cells were exposed to 0–1 μM T4, 0–25 nM T3, or 0–25 nM rT3 for 15 h followed by RNA preparation.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

At the end of the TH treatments, RNA and cDNA were prepared from cells using the Fastlane Cell cDNA Kit (Qiagen), following the manufacturer's instructions. The relative expression levels of MDR1, CYP3A4, and UGT1A1 mRNAs were measured using quantitative real-time PCR and the comparative C_t method, described by Applied Biosystems. The relative level of β-actin was also measured as an endogenous reference. The primers and probes for the MDR1, CYP3A4, and UGT1A1 cDNAs were all TaqMan Gene Expression Assays or Assays-on-Demand products from Applied Biosystems. For the normalization of the expression data, the Pre-Developed TaqMan Assay Reagents Endogenous Control human β-actin Kit (VIC-labeled probe; Applied Biosystems) was used. Real-time PCR was performed in 25-μl reactions using the Prism 7000 Sequence Detection System (Applied Biosystems), and the data were analyzed according to the manufacturer's guidelines. Each value represents the mean ± SD of four independent experiments. The fold induction was calculated relative to the untreated cells for each gene.

Plasmids

The *MDR1* 5′-flanking region (−10082/+117) was amplified using the TaKaLa LA PCR Kit (Takara) and genomic DNA prepared from LS180 cells as a template and the primer pairs 5′-CTGGTACCTTGTCATTGTTGAGAAGACGCTG-3′ and 5′-ACGCGTCGACG AACGGCCACCAAGACGTGAA-3′, which include the Kpn I and Sal I sites for cloning. The amplified fragment was digested by Kpn I and Sal I enzymes and ligated to the Kpn I/Xho I site of firefly luciferase rapid response reporter vector pGL4.12 (Promega), resulting in pMD10082L. The plasmids pMD7970L, pMD7145L, and pMD457L were constructed by deleting the Nhe I, Nhe I/Xcm I, and Sph I fragments from pMD10082L, respectively. pMD*824L was constructed by deleting the Xcm I/Sph I fragment from pMD7970L. pMD*824Δ90L, pMD*824Δ153L, and pMD*824Δ214L were constructed using pMD*824L as a template and the In-Fusion Dry-Down PCR Cloning Kit (Clontech) with a reverse common primer 5′-TCTG CAGTGGTCTTTCTTCAG-3′ and forward individual primers 5′-AAAAGGGGATGCTAG ACGTTACCTCATTGAACTAACTTGA-3′, 5′-AAAAGGGGATGCTAGGTTCAAAGTCTATGA ATCATAAAACGATAA-3′, and 5′-AAAAGGGGATGCTAGAGCTCTGCAATTCAAAAGCCA-3′, respectively.

Mutations at the half-sites of the direct repeats were introduced into the pMD*824Δ90L reporter plasmid using the Quick-Change Multi Site-Directed Mutagenesis Kit (Stratagene), according to the manufacture's instructions, with the following oligonucleotides used alone or in combination:

M1, 5′-AAGGGGATGCTAGACGTTTTCTCATTGAACTAACTTGAC-3′;
M2, 5′-GCTAGACGTTACCTCATTGTTCTAACTTGACCTTGCTCCTG-3′;
M3, 5′-GAACTAACTTGTTCTTGCTCCTGGG-3′;
M4, 5′-GACCTTGCTCCTGGGAGAGAGAACATTTGAGATTAAACAAG-3′;
M7, 5′-CCTGGGAGAGAGTTCATTTGAGATGGAACAAGTTCAAAGTCTATG-3′; and
M12, 5′-GAGAGAGTTCATTTGAGATTAAACAAGTTTTAAGTCTATGAATC-3′.

All the mutations were verified using DNA sequencing on an Applied Biosystems 3730 sequencer (Applied Biosystems). Human TRβ (TRβ1) cDNA in a pME18SFL3 vector was purchased from Toyobo Co., Ltd. EcoR I and Not I fragments, including the full-length TRβ cDNA, was subcloned into pcDNA3.1 (Invitrogen), resulting in the TRβ expression plasmid pcTRβ. The expression plasmid encoding human RXRα cDNA (pcDNA3.1-hRXRα) was a generous gift from Dr. Shuichi Koizumi (Yamanashi University, Japan).

Transient transfection and luciferase-reporter gene assays

Cells were seeded into 96-well plates and grown until 70–80% confluence, then transiently transfected using HilyMax transcription regent (Dojindo Laboratories, Japan) at a reagent:DNA ratio of 5:1, according to the manufacturer's instructions. One hundred nanograms/well of the luciferase-reporter plasmid to be examined was transfected with 10 ng/well of pcTRβ or pcDNA3.1 and 10 ng/well of pGL4.74 (Promega), a plasmid encoding the *Renilla reniformis* luciferase and used to normalize the transfection efficiency, in 100 μl/well of medium. Twenty-four hours after transfection, the cells were treated with 50 nM of T3. Firefly and *Renilla* luciferase activities were measured 3.5 h after treatment using the Dual-Glo Luciferase kit (Promega) in a Wallac 1420 ARVOsx Multilabel counter (PerkinElmer Life Science). The transfection efficiency of the firefly luciferase activity was normalized against the activity of the internal *Renilla* luciferase activity. The fold induction was calculated as a ratio of (the activity from T3-treated cells)/(the activity from untreated cells). Each value represents the mean ± SD of four independent experiments, in each of which the fold induction value was the ratio of the mean activity from four wells of T3-treated cells divided by the mean activity from four wells of untreated cells.

Chromatin immunoprecipitation (ChIP) assays

Caco-2 cells were transiently transfected with 6 μg/dish (100-mm) of pcTRβ and pcDNA3.1-hRXRα. After incubation for 40 h, the culture medium was changed to phenol red-free DME medium supplemented with 10% dextran-coated charcoal-treated fetal bovine serum. After incubation for 4 h, the cells were used for ChIP assays by using ChIP-IT Express Enzymatic Kit (Active Motif, Inc.) according to the manufacturer's instructions. Immunoprecipitation of the chromatin complexes were performed with an anti-human TRβ1 mouse monoclonal antibody (sc-738; Santa Cruz Biotechnology, Inc.) and anti-human RXRα mouse monoclonal antibody (PP-K8508-00; Perseus Proteomics, Inc.). As a negative control, anti-human PXR mouse monoclonal antibody (PP-H4417-00; Perseus Proteomics, Inc.) was used because PXR is not expressed in the Caco-2 cells. PCR was performed with 5 μl (from a total of 100 μl) of eluted immunoprecipitate or of 1.0% input (chromatin taken before immunoprecipitation) in a total volume of 25 μl for 30 cycles. The set of primers used for the amplification of −7886 to −7730 bp (TR-binding region) were: forward 5′-GAGTGAACGTTACCTCATTGAAC-3′ and reverse 5′-CCGAAATGGCTTTTG AATTG-3′. The set of primers used for the amplification of −9889 to −9762 bp (negative control region) were: forward 5′-AAATATGAGATGCATAGAGCC-3′ and reverse 5′-AACCTTCTTACTCTACTATAGTC-3′. Cycling conditions were 94 °C for 5 min, followed by 30 cycles at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 15 s. PCR products were resolved on 2.2% FlashGel DNA Cassettes (Lonza Rockland, Inc.).