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Sterol regulatory element-binding proteins are transcriptional regulators of the thyroglobulin gene in thyroid cells

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ABSTRACT

The genes encoding sodium/iodide symporter (NIS) and thyroid peroxidase (TPO), both of which are essential for thyroid hormone (TH) synthesis, were shown to be regulated by sterol regulatory element-binding proteins (SREBP)-1c and -2. In the present study we tested the hypothesis that transcription of a further gene essential for TH synthesis, the thyroglobulin (TG) gene, is under the control of SREBP. To test this hypothesis, we studied the influence of inhibition of SREBP maturation and SREBP knockdown on TG expression in FRTL-5 thyrocytes and explored transcriptional regulation of the TG promoter by reporter gene experiments in FRTL-5 and HepG2 cells, gel shift assays and chromatin immunoprecipitation. Inhibition of SREBP maturation by 25-hydroxycholesterol and siRNA-mediated knockdown of either SREBP-1c or SREBP-2 decreased mRNA and protein levels of TG in FRTL-5 thyrocytes. Reporter gene assays with wild-type and mutated TG promoter reporter truncation constructs revealed that the rat TG promoter is transcriptionally activated by nSREBP-1c and nSREBP-2. DNA-binding assays and chromatin immunoprecipitation assays showed that both nSREBP-1c and nSREBP-2 bind to a SREBP binding motif with characteristics of an E-box SRE at position -63 in the rat TG promoter. In connection with recent findings that NIS and TPO are regulated by SREBP in thyrocytes the present findings support the view that SREBP are regulators of essential steps of TH synthesis in the thyroid gland such as iodide uptake, iodide oxidation and iodination of tyrosyl residues of TG. This moreover suggests that SREBP may be molecular targets for pharmacological modulation of TH synthesis.

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1. Introduction

The key function of the thyroid gland is to produce the thyroid hormones (TH) thyroxine (tetraiodothyronine, T_4) and triiodothyronine (T_3), which are essential for development, growth and regulation of numerous metabolic pathways in vertebrates [1]. TH synthesis requires an adequate iodide supply through the diet, because iodine is an indispensable component of TH, and efficient synthesis machinery with appropriate controls [2]. The TH synthesis machinery includes several steps taking place in the angiofollicular units of the thyroid. These morphological-functional units of the thyroid consist of the thyroid follicles, which are formed by a single layer of polarized thyrocytes surrounding the colloid-containing follicular lumen, and the microvasculature [3,4]. TH synthesis starts with the active uptake of the absorbed iodide from the blood across the basolateral membrane of the thyrocyte by the sodium/iodide symporter (NIS) [5], followed by transcellular transport and export of iodide across the apical thyrocyte membrane via pendrin [6]. At the apical membrane-colloid interface, iodide is oxidized by hydrogen peroxide via the activity of thyroid peroxidase (TPO) leading to the attachment of iodine to selected tyrosyl residues of thyroglobulin (TG) [7]. The TG is a 660 kDa glycoprotein secreted from the thyrocyte into the follicular lumen [7,8]. The initial iodination of TG leads to mono- and diiodotyrosine (MIT and DIT, respectively), and enzymatic coupling of the iodotyrosyl residues by TPO produces the TH [9]. The critical role of NIS, TPO and TG for TH synthesis is shown by the fact that genetic abnormalities in any of these key proteins leads to severe congenital hypothyroidism and goiter formation [10–12].

Thyrotropin (TSH) secreted from the pituitary gland is the primary regulator of all steps involved in TH synthesis [13] and mediates its effects on the thyrocyte through the TSH receptor (TSHR)/cAMP/protein kinase A (PKA) pathway [14,15]. Activation of this pathway stimulates expression of NIS, TPO and TG at the transcriptional and/or posttranslational level and increases iodide accumulation and iodination in the thyrocyte [16–21]. TSH-dependent transcriptional regulation of NIS, TPO and TG has been demonstrated to involve binding sites for thyroid transcription factor (TTF)-1, forkhead box E1 (FOXE1, also named TTF-2), paired box-8 (PAX-8) and/or cAMP response element (CRE)-binding proteins [22–26]. Despite the outstanding role of the TSH-dependent signaling pathway in regulating genes involved in TH synthesis, it was reported that NIS, TPO and TG are also transcriptionally regulated by

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TSH-independent pathways like the NF-kB pathway or non-TSH signals like lipopolysaccharide [27–29]. In this context it was moreover shown that NIS and TPO are up-regulated by sterol regulatory element-binding proteins (SREBP)-1c and -2 via specific SREBP binding motifs, called sterol response elements (SRE), in their regulatory region [30,31]. SREBP were initially discovered as master transcription factors of cholesterol and fatty acid synthesis [32]. Interestingly, expression and activation of SREBP was found to be stimulated by TSH in thyrocytes, an effect that mechanistically explains earlier observations that TSH stimulates the synthesis of cholesterol and fatty synthesis in thyrocytes [33–35]. These lipids are important membrane constituents and required for thyrocyte growth and proliferation both of which are stimulated by TSH. Thus, these observations indicate that TSH coordinates lipid and TH synthesis in growing and proliferating thyrocytes through SREBP. In light of the recent identification of SREBP as transcriptional regulators of two key genes involved in TH synthesis, NIS and TPO, we hypothesized that TG gene transcription is also under the control of SREBP. To test this hypothesis, we studied the influence of inhibition of SREBP maturation and SREBP knockdown on TG expression in the Fisher rat thyroid cell line-5 (FRTL-5) and explored transcriptional regulation of the TG 5′-flanking region by reporter gene experiments, gel shift assays and chromatin immunoprecipitation.

2. Material and methods

2.1. Cell culture

The FRTL-5 and the human hepatoma G2 (HepG2) cell lines were obtained from CLS (Eppelheim, Germany) and DSMZ (Braunschweig, Germany), respectively. HepG2 cells were cultured with RMPI 1640 medium supplemented with 10% FBS and FRTL-5 cells with Ham's F12 medium supplemented with 5% NCS and a six-hormone mixture (“6 hormone medium”) as described in our recent publication [30]. Medium was changed every 2 days. After reaching a confluence of 70–80%, the cells were either sub-cultivated or used for experiments.

2.2. Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell viability in response to 25-hydroxycholesterol (25-HC). FRTL-5 cells were seeded in 96-well culture plates at a density of 5 x 10^4 cells per well and treated with the indicated concentrations of 25-HC in either 5 hormone (without TSH) or 6 hormone (with TSH) medium for 24 h. Control cells were treated with the same concentration of vehicle (ethanol; 0.1%). The MTT assay was performed as described recently [36], with the exception that FRTL-5 cells were incubated with the MTT solution for 4 h. Cell viabilities of treated cells are presented relative to that of control cells, which was set to 100%.

2.3. RNA isolation and quantitative PCR (qPCR)

For qPCR experiments, FRTL-5 cells were seeded in 24-well culture plates at a density of 1 x 10^5 cells per well and treated as indicated. Following treatment, total RNA was extracted from cells using Trizol reagent (Life Technologies, Darmstadt, Germany) according to the manufacturer's protocol. cDNA synthesis and qPCR using gene-specific primers synthesized by Eurofins Genomics (Ebersberg, Germany) were performed as described recently in detail [30]. Features of gene-specific primers are shown in Table 1. Relative gene expression levels were calculated with the $2^{-\Delta Ct}$ equation [37] and normalized by the GeNorm normalization factor based on the geometric mean of multiple reference genes [38]. In the present study, the stability score M of all four tested potential reference genes (ACTB, RPL13, TOP1, YWHAZ) indicated suitability for normalization. Normalized mRNA concentration of treated cells is presented relative to that of control cells, which was set to 1.0.

2.4. Immunoblotting

For immunoblotting experiments, FRTL-5 cells were seeded in 6-well culture plates at a density of 3 x 10^5 cells per well and treated as indicated. Following treatment, cells were lysed with radioimmunoprecipitation assay lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate] containing protease inhibitors (Sigma-Aldrich Chemie GmbH, Germany). Determination of protein content, protein separation by SDS-PAGE, electrotransfer to nitrocellulose membranes and blocking of membranes were carried out as described recently [30]. Afterwards, membranes were incubated overnight at 4 °C with primary anti-TG antibody (rabbit polyclonal; 1:500 dilution; Abcam, Cambridge, UK) or anti-β-actin antibody (mouse monoclonal; 1:10.000 dilution; Abcam) as a reference protein for normalization. The membranes were washed, and then incubated with a horseradish peroxidase-conjugated secondary anti-IgG antibody (rabbit polyclonal or mouse monoclonal; 1:10.000 dilution; Sigma-Aldrich and Abcam, respectively) at room temperature. Blot development and detection by chemiluminescence were performed as described recently [30]. For calculation of normalized protein concentrations, the band intensity of TG was normalized by that of β-actin. Normalized protein concentration of treated cells is presented relative to that of control cells, which was set to 1.0.

2.5. RNA interference

FRTL-5 cells with a targeted knockdown of SREBP-1c or SREBP-2 were generated by transfection of gene-specific Stealth RNAi molecules (from Invitrogen, Karlsruhe, Germany) using LipofectAMINE 2000 (Invitrogen) as described recently [30,31]. Following transfection, FRTL-5 cells were incubated with 6 hormone medium for 24 h and then total RNA extraction and cell lysis, respectively, was carried out as described above.

Table 1
Characteristics of gene-specific primers used for qPCR.

Gene symbol	Primer sequence (forward, reverse)	Product size (bp)	NCBI GenBank
Reference genes			
ACTB	GACCTCTATGCCAACACAGT CACCAATCCACACAGAGTAC	154	NM_031144
RPL13	CTTAAATTGGCCACGCAGCT CTTCTCAACGTCTTGCTCTG	198	XR_086310
TOP1	GAAGAACGCTATCCAGAAGG GCTTTGGGACTCAGCTTCAT	137	NM_022615
YWHAZ	GACGGAAGGTGCTGAGAAA GCAGCAACCTCAGCCAAGT	198	NM_013011
Target genes			
TG	GTTCCTACGTGTACTAGTGAG, CATACTGGAGTTGGAGAGCAG	196	NM_030988
PAX8	CCTTACTCAACAGTACCCTGG AGCTAGAACTGGAGAGCTCTG	162	NM_031141
TTF1	GCATGAATATGAGCGGCATGG ACTTCTGCTGCTTGAAGCGTC	153	NM_013093
TTF2	GAAGTGGCAGAACAGCATCC AGCTGCCGCTCTCGAACATG	139	NM_138909

Abbreviations of gene symbols: ACTB, β-actin; RPL13, ribosomal protein L13; TOP1, topoisomerase (DNA) I; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; TG, thyroglobulin; PAX8, paired box 8; TTF1 (or NKX2-1), NK2 homeobox 1; TTF2 (or FOXE1), Forkhead box E1.

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