Thyroid stimulating hormone suppresses the expression and activity of cytosolic sulfotransferase 1a1 in thyrocytes

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Abstract. Sulfonation is an important step in the metabolism of dopamine, estrogens, dehydroepiandrosterone, as well as thyroid hormones. However, the regulation of cytosolic sulfotransferases in the thyroid is not well understood. In a DNA microarray analysis of rat thyroid FRTL-5 cells, we found that the mRNA expression of 10 of 48 sulfotransferases was significantly altered by thyroid stimulating hormone (TSH), with that of sulfotransferase family 1A member 1 (SULT1A1) being the most significantly affected. Real-time PCR and Western blot analyses revealed that TSH, forskolin and dibutyryl cyclic AMP significantly suppressed SULT1A1 mRNA and protein levels in a time- and concentration-dependent manner. Moreover, immunofluorescence staining of FRTL-5 cells showed that SULT1A1 is localized in the perinuclear area in the absence of TSH but is spread throughout the cytoplasm with reduced fluorescence intensity in the presence of TSH. Sulfotransferase activity in FRTL-5 cells, measured using 3'-phosphoadenosine-5'-phosphosulfate as a donor and p-nitrophenol as an acceptor substrate, was significantly reduced by TSH. These findings suggest that the expression and activity of SULT1A1 are modulated by TSH in thyrocytes.

Key words: Thyroid, Sulfotransferase, Sulfotransferase family 1A member 1 (SULT1A1), Thyroid stimulating hormone, FRTL-5

THYROID FOLLICLES. consisting of a monolayer of thyrocytes surrounding a colloid-containing lumen, are the minimum functional unit for the production, storage and secretion of thyroid hormones (THs), i.e., thyroxine (T4) and triiodothyronine (T3) [1]. To produce THs, thyrocytes concentrate iodide via solute carrier family 5 member 5 (SLC5A5; sodium-iodide symporter: NIS) from the bloodstream. Iodine is then transported into the follicular lumen by SLC26A4 (pendrin) and SLC26A7. After secretion into the follicular lumen, thyroid peroxidase (TPO) catalyzes the binding of thyroglobulin (Tg) to select tyrosine residues to form 3-iodothyronine (or monoiodotyrosine: MIT) and 3,5'-diiodothyronine (DIT), followed by the coupling of two iodotyrosines to form T3 and T4 [2]. The iodinated Tg is stored in the colloid and re-internalized into thyrocytes via endocytosis, thereby liberating THs upon thyroid stimulating hormone (TSH) stimulation [3, 4]. THs are secreted in the bloodstream via SLC16A2 (or monocarboxylate transporter symporter 8: MCT8) expressed on the basolateral plasma membrane of thyrocytes [5, 6].

During this process, newly synthesized Tg undergoes extensive post-translational modifications including sulfonation [7-9]. Sulfonation is performed by a class of enzymes called sulfotransferases, which catalyze the transfer of negatively charged sulfate groups from the universal sulfate donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) onto their substrates. Depending on the substrate, sulfotransferases are classified as tyrosylprotein sulfotransferase (TPST), carbohydrate...
sulfotransferase (CHST), heparan sulfotransferase (NHSST) [10], galactose-3-O-sulfotransferase (Gal3ST) [11], N-deacetylase and N-sulfotransferase (NdST) [12] and cytosolic sulfotransferase (SULT) [13]. Sulfonation is a common post-translational modification of Tg found throughout the vertebrate phylum, suggesting that it was formed early in evolution of the thyroid gland [14]. On the other hand, sulfotransferases play important roles in the metabolism of endogenous compounds, such as catecholamines, steroid hormones and THs, as well as xenobiotics and some drugs [15]. Sulfonation contributes to TH metabolism and homeostasis in both thyroid and extrathyroidal tissues. For instance, sulfonation of THs accelerates deiodination by iodothyronine deiodinase (DIO) 1 and their subsequent degradation and excretion [16].

Although SULT1A1 seems to play a key role in TH synthesis and metabolism in thyrocytes [17], regulation of sulfonation in the thyroid is still poorly understood. In the present study, to shed some light on the effect of TSH on the regulation of sulfonation in the thyroid, we elucidated the comprehensive transcriptional profile of sulfotransferases using rat thyroid FRTL-5 cells.

**Materials and Methods**

**Cell culture and treatment**

Rat thyroid FRTL-5 cells were seeded into 6 well plate (Thermo Fisher Scientific, Waltham, MA, USA) and cultured in Coon’s modified Ham’s F-12 medium supplemented with 5% bovine serum (Invitrogen, Waltham, MA, USA) and a mixture of six hormones (10 μg/mL insulin, 0.36 ng/mL hydrocortisone, 5 μg/mL transferrin, 10 ng/mL somatostatin, 2 ng/mL glycy1-L-histidyl-L-lysine acetate and 1 μM/mL TSH-H) as described previously [18-20]. The cells were initially grown in a fully supplemented medium for 2 days and then transferred to a medium without TSH for 5 days. Cells were then treated with 1 μM/mL TSH, 1 μM forskolin (Sigma-Aldrich, St. Louis, MO, USA) or 500 μM dibutyryl cAMP (dbcAMP; Sigma-Aldrich) for 6, 12, 24 or 48 h, or they were treated with 0.01, 0.1, 1 or 10 μM/mL TSH; 0.01, 0.1, 1 or 10 μM forskolin; or 50, 100, 200 or 500 μM dbcAMP for 24 h.

**DNA microarray analysis**

FRTL-5 cells grown in the absence of TSH were treated with the medium specified above containing 1 μM/mL TSH for 24 h. Total RNA was purified using the RNAse Plus Mini Kit (Qiagen, Hilden, Germany), and 1 μg total RNA was amplified using the Amino Allyl aRNA amplification kit (#1753, Ambion, Austin, TX, USA) as described previously [21]. The DNA microarray results were analyzed by TORAY Industries, Inc. (Tokyo, Japan). Briefly, Cy5 labeling of cDNA was performed using Amersham Vy5 Mono-Reactive Dye (#PA25001, GE Healthcare, Buckinghamshire, UK). After purification, 1 μg of each Cy5-labeled sample was hybridized to the array in a hybridization chamber (#TX711, Takara Bio, Shiga, Japan) for 16 h at 37°C. The arrays were scanned using the 3D-Gene Scanner 3000 (TORAY Industries).

**Total RNA isolation and real-time PCR**

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen), and reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) as described previously [22, 23]. Quantitative real-time PCR was performed using the Fast SYBR Green Master Mix (Applied Biosystems) and run on the Thermal Cycler Dice Real Time System III (Takara Bio) according to the manufacturer’s instructions. Briefly, 5 ng cDNA was mixed with 10 μL 2× Fast SYBR Green Master Mix and the primers, then incubated for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 60 s at 60°C and 1 cycle of 15 s at 95°C, 30 s at 60°C and 15 s at 95°C. The relative mRNA levels were normalized with respect to *Gapdh* using the ΔΔCt method as described previously [24, 25]. The sequences of the PCR primers are listed in Supplementary Table 1.

**Protein preparation and Western blot analysis**

Cellular protein was extracted and analyzed as previously described [23, 26, 27]. Briefly, FRTL-5 cells were washed 2 times with ice-cold phosphate buffered saline (PBS). Cells were collected and lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4 and 1 μg/mL leupeptin. The lysates were centrifuged at 12,000 g at 4°C for 10 min to recover the supernatant, and the protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Ten micrograms of each protein sample were heated in sodium dodecyl sulfate (SDS) sample loading buffer containing 1% 2-mercaptoethanol at 95°C for 5 min and loaded on a NuPage 4–12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes using iBlot gel transfer stacks (Invitrogen) with Novex iBlot gel transfer system (Invitrogen). The membrane was washed with PBS containing 0.1% Tween 20 (PBST), placed in blocking buffer (PBST containing 5% nonfat milk) for 1 h at room temperature, and then
incubated overnight at 4°C with rabbit anti SULT1A1 (1:1,000; St John’s Laboratory Ltd, London, UK) or mouse anti β-actin (1:10,000; Cell Signaling Technology). After washing with PBST, the membranes were incubated with horseradish peroxidase (HRP)-labelled horse anti-rabbit IgG (1:1,000; Cell Signaling Technology) or HRP-labelled anti-mouse IgG (1:10,000; Cell Signaling Technology) for 1 h at room temperature. The chemiluminescence signal was developed using ImmunoStar LD reagents (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and the images were scanned with the C-Digit blot scanner (LI-COR, Lincoln, NE, USA).

**Immunofluorescence staining and confocal laser scanning microscopy**
FRTL-5 cells were seeded in the 4-well chamber slides (Thermo Fisher Scientific) for 24 h. The cells were then maintained in medium without TSH for 5 days, followed by treatment with 1 mU/mL TSH for up to 120 h. After culturing, the cells were washed with PBS, fixed using 10% neutral buffered formalin for 10 min at room temperature, and then permeabilized with 0.1% Triton X-100 (Thermo Fisher Scientific) in PBS for 10 min at room temperature. After treating with 3% bovine serum albumin (Sigma-Aldrich) in PBS for 1 h, the cells were incubated overnight at 4°C with rabbit anti SULT1A1 (1:200; St John’s Laboratory Ltd), washed with PBST and then incubated with Alexa Fluor 594-labeled chicken anti-rabbit IgG (1:1,000; Cell Signaling Technology), followed by nuclear staining with Hoechst 33342 (Cell Signaling Technology). Immunofluorescence was visualized and the images were captured with the confocal laser scanning microscope FV10i-DOC (Olympus, Tokyo, Japan).

**Measurement of sulfotransferase activity**
FRTL-5 cells were maintained in 6-well plates in medium without TSH for 5 days, after which the cells were exposed to TSH for 24, 48 or 72 h. Cells were washed with ice-cold Tris-MgCl₂ buffer containing 50 mM Tris-HCl (pH 7.5) and 100 mM MgCl₂ and subsequently lysed in cell lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM MgCl₂ and 1% NP40). Cells were then collected using a disposable cell lifter and centrifuged at 12,000 g at 4°C for 10 min. The supernatant was recovered and dialyzed against Tris-MgCl₂ buffer at 4°C for 2 h using a dialysis membrane with a 15–50 Å average pore size, 14,000 molecular weight cut-off, and 20 μm thickness (FUJIFILM Wako Pure Chemical Corporation). The protein concentrations were measured using the DC Protein Assay Kit, and 10 μg of each protein sample was used to measure sulfotransferase activity using the Universal Sulfotransferase Activity Kit (R&D Systems, Minneapolis, MN, USA). To measure sulfotransferase activity, 10 μg protein was mixed with a reaction mixture of 1 mM PAPS, 5 mM p-nitrophenol (FUJIFILM Wako Pure Chemical Corporation) and 500 ng 3'-phosphatase in a final volume of 50 μL and incubated for 20 min at room temperature. In this reaction, sulfotransferases catabolize PAPS to 3'-phosphodadenosine-5'-phosphate (PAP), and a phosphate in the 3' position of PAP, but not of PAPS, is released by 3'-phosphatase. The amount of inorganic phosphate released, which is proportional to the amount of PAP produced, was measured using malachite green at an absorbance of 620 nm on the Multiskan FC spectrophotometer (Thermo Fisher Scientific) [28].

**Statistical analysis**
All experiments were performed using triplicate samples and were repeated at least three times. Results are expressed as the mean ± standard deviation. Significant differences were determined by one-way ANOVA followed by Dunnett’s post hoc test. p < 0.05 was considered to represent significance.

**Results**

**Transcriptional profiling of sulfotransferases in FRTL-5 cells before and after TSH stimulation**
To assess the potential effects of TSH on the mRNA expression of sulfotransferases in thyroid cells, we reanalyzed our DNA microarray data obtained previously in rat thyroid FRTL-5 cells treated with or without TSH [22]. Overall, at 24 h after stimulation, TSH downregulated and upregulated the expression of 1,211 and 1,458 genes more than two-fold, respectively. Among 48 sulfotransferases detected in FRTL-5 cells, the expression levels of sulfotransferase5a1 (Sult5a1), tyrosylprotein sulfotransferase2 (Tpst2), carbohydrate sulfotransferase3 (Chst3) and galactose-3-O-sulfotransferase4 (Gal3st4) were upregulated more than two-fold, while sulfotransferase1a1 (Sult1a1), sulfotransferase1d1 (Sult1d1), tyrosylprotein sulfotransferase1 (Tpst1), carbohydrate sulfotransferase10 (Chst10), carbohydrate sulfotransferase12 (Chst12) and heparan sulfate 3-O-sulfotransferase1 (Hs2st1) were downregulated more than two-fold (Supplementary Table 2, Fig. 1).

Among these, Sult1a1 was the third most abundantly expressed sulfotransferase in FRTL-5 cells in the absence of TSH, and its expression was the most prominently affected by TSH (Supplementary Table 2). Although SULT1A1 is expressed in many different tissues, it is highly expressed in the kidney, lung, brain, skin, platelets, gastrointestinal tract and liver [15]. SULT1A1 exhibits a broad substrate range, but it is
especially specific to small phenolic groups, e.g., p-nitrophenol. Stimulation with 1 μM TSH for 24 h strongly suppressed the Sult1a1 mRNA level to 6% of its original level in FRTL-5 cells (Supplementary Table 2). We therefore further investigated the effect of TSH on SULT1A1 in FRTL-5 cells.

**TSH/TSH receptor (TSHR) signaling suppressed Sult1a1 mRNA expression in FRTL-5 cells**

We first examined the effect of TSH on the Sult1a1 mRNA level using real-time PCR. FRTL-5 cells were cultured in medium without TSH for 5 days, after which they were cultured in fresh medium supplemented with 0.01, 0.1, 1 or 10 μM TSH for up to 24 h. The results clearly showed that TSH significantly suppressed the Sult1a1 mRNA level in a concentration-dependent manner (Fig. 2A, left panel). Stimulating cells with 1 μM TSH for various times showed that TSH inhibited Sult1a1 mRNA expression in a time-dependent manner, with the Sult1a1 mRNA level decreasing to 15% of the original level as soon as 6 h and then to 2% at 24 h (Fig. 2A, right panel). We next examined whether the TSH/TSHR/cAMP signaling cascade is involved in the regulation of Sult1a1 mRNA expression, by evaluating the effects of forskolin (which increases the intracellular cAMP concentration by stimulating adenylate cyclase [29]) and dbcAMP (a cell-permeable cAMP analog) on

Sult1a1 mRNA expression. As a result, 0.01, 0.1, 1 or 10 μM forskolin and 50, 100, 200 or 500 μM dbcAMP significantly suppressed the Sult1a1 mRNA level in both a concentration- and time-dependent manner (Fig. 2B and 2C). These data indicate that downstream signaling of TSHR is responsible for the suppression of Sult1a1 mRNA expression in thyroid cells. Real-time PCR was also performed on several other sulfotransferases whose
expression was affected by TSH according to the DNA microarray analysis. However, the effect of TSH on those sulfotransferases was not as significant as the effect on Sult1a1 according to real-time PCR (Supplementary Fig. 1).

**TSH/TSHR signaling suppressed Sult1a1 protein expression in FRTL-5 cells**

We next examined whether the SULT1A1 protein level was affected by TSH/TSHR signaling. We performed Western blot analysis using a specific antibody against SULT1A1. In accordance with the changes in mRNA expression, the protein level of SULT1A1 was also decreased by TSH, forskolin and dbcAMP in both a concentration- and time-dependent manner (Fig. 3A–3C). Densitometric analysis of the specific bands confirmed a significant reduction in the SULT1A1 protein level by TSH and its downstream effectors (Fig. 3).

We also performed immunofluorescence staining of SULT1A1 using the same antibody used for Western blot analysis and observed immunofluorescence by confocal laser scanning microscopy. In the absence of TSH, SULT1A1 was observed in the cytoplasm in a granular pattern, especially in the peri-nuclear area. However, in the presence of TSH, the fluorescence intensity was reduced in a time-dependent manner, which concords with the decreased SULT1A1 protein level demonstrated by Western blot analysis, and SULT1A1 localization was spread throughout the cytoplasm (Fig. 4). These results demonstrate that TSH utilized the cAMP signaling cascade to regulate the expression and subcellular localization of SULT1A1.

**TSH decreased sulfotransferase activity in FRTL-5 cells**

Finally, we examined the effect of TSH on the activity of sulfotransferases in FRTL-5 cells. FRTL-5 cells were cultured in the absence of TSH for 5 days and then switched to fresh medium containing 1 mU/mL TSH for up to 72 h. Cells were then washed with Tris-MgCl₂, and lysed in cell lysis buffer at 4°C. The cell lysate was centrifuged, and the supernatant was dialyzed against Tris-MgCl₂ at 4°C for 2 h. The sulfotransferase activity was measured using PAPS as a sulfate donor and p-nitrophenol as an acceptor. The level of phosphate released from the 3’ position of PAP by 3’-phosphatase was measured as the amount of PAP produced by sulfotransferase activity. The results showed that sulfotransferase activity was significantly decreased to 63% and 68% at 48 and 72 h after TSH stimulation, respectively, relative to the level before adding TSH (Fig. 5), indicating that TSH suppressed overall sulfotransferase activity in FRTL-5 cells.

![Fig. 3 TSH, forskolin and dbcAMP suppressed SULT1A1 protein expression in FRTL-5 cells](image-url)
The cells were initially grown in a fully supplemented medium for 2 days and then transferred to a medium without TSH for 5 days and then switched to the medium containing 1 mU/mL TSH for the indicated times. Cells were fixed and subjected to immunofluorescent staining of SULT1A1 (red) and nuclear (blue) staining using Hoechst 33342. Immunofluorescence was visualized and the images were captured with the confocal laser scanning microscope FV10i-DOC. Scale bar: 20 μm.

**Discussion**

Sulfonation is intricately involved in the regulation of TH biosynthesis and metabolism. To elucidate the mechanisms underlying the regulation of sulfonation in thyrocytes, we comprehensively examined the expression levels of sulfotransferases in FRTL-5 cells before and after TSH stimulation using DNA microarray data that we obtained previously [22]. Transcriptional profiling revealed that Sult1a1 was one of the most abundantly expressed cytosolic sulfotransferases in FRTL-5 cells and the sulfotransferase most remarkably affected by TSH in terms of expression, which persuaded us to further investigate its regulation by TSH. In agreement with the DNA microarray data, we showed that both the mRNA and protein levels of SULT1A1 were suppressed by TSH and the overall sulfotransferase activity in FRTL-5 cells was significantly decreased by TSH.

Sulfonation is an important late post-translational modification of Tg protein molecules in the Golgi apparatus [30]. TSH might act at different steps of Tg sulfonation at the sites of carbohydrate units and peptide chains by modulating the substrates and/or enzymes [31].
Tyrosine residues in the protein core of Tg are also substrates for sulfonation, which is likely involved in hormonogenesis and regulated by TSH [17]. It was found that the same tyrosine residues within Tg molecules undergo both iodination and sulfation, and that the most prevalent site of T4 formation (tyrosine 5 of porcine Tg) is sulfonated and may act as a signal for iodination or the coupling reaction [32, 33]. Whether or not SULT1A1 is involved in these sulfonation reactions needs clarification in future studies.

Human SULT1A1 is ubiquitously expressed in the thyroid and many other tissues, and it catalyzes the sulfonation of simple phenols, such as 1-naphthol and p-nitrophenol [15]. Catalytic studies suggest that SULT1A1 plays a role in the sulfonation of THs, contributing to the metabolism of THs [34]. Sulfonation of T4 blocks the outer ring deiodination of T4 sulfate by iodothyronine deiodinases 1 (DIO1), effectively preventing active T3 production and resulting in irreversible inactivation of T4 to produce more rT3 [35]. T3 sulfate and 3,3'-T2 provide better substrates for DIOs and further facilitate their deiodination [36]. Sulfonation thus provides a unique pathway that accelerates the deiodination of THs, which may titrate the levels of biologically active hormones and/or efficiently reutilizes iodide from excessively synthesized THs.

SULT1A1 is likely the predominant sulfotransferase to sulfonate 3,3'-T2 in the human thyroid, as SULT1A1 activity is highly specific to 3,3'-T2 [34]. Human thyroid SULT1A1 promotes deiodination by sulfonating 3,3'-T2 and may also be involved in the reutilization of iodide and subsequent production of THs. Intriguingly, when SULT1A1 activity was measured using p-nitrophenol as a substrate, the addition of T4, T3, rT3 and 3,3'-T2 each inhibited SULT1A1 activity with different efficacies [34]. This result indicates that the ratio of intrathyroidal THs to iodothyronines may also affect their sulfonation and subsequent metabolism via a feedback mechanism. In support of this idea, increased levels of serum and urine T3 sulfate were found in a patient treated with T4 [37]. Thus, intrathyroidal sulfonation may be an essential step in the metabolism of excess THs that is not secreted but degraded for iodine recycling.

TSH leads to activation of TSHR, a G-protein-coupled receptor, thereby stimulating secondary messenger pathways involving predominantly cAMP and inositol 1,4,5-trisphosphate (IP3) [38, 39]. The key signaling pathway activated by TSH is mediated via Gs which leads to an increase in intracellular cAMP. The TSH/TSHR/cAMP pathway suppressed SULT1A1 expression in FRTL-5 cells and inhibited the overall sulfotransferase activity in the cells. Since sulfonation is thought to be positively associated with the metabolism of THs, TSH at physiological levels likely prevents active degradation of THs in the thyroid. Although the decrease in sulfotransferase activity induced by TSH was not as significant as the dramatic decrease in SULT1A1 protein expression in FRTL-5 cells, this may be because the measured activity reflects that of all sulfotransferases, the activities of which may not be decreased by TSH. Since SULT1A1 was the most sensitive sulfotransferase to TSH in FRTL-5 cells, further investigation is needed to elucidate its physiological functions in the thyroid. Nevertheless, our findings indicate that TSH contributes to the homeostasis of THs in thyrocytes by affecting the sulfonation status via various sulfotransferases. Elucidation of the function and regulation of TH sulfonation in thyrocytes would broaden our understanding of the regulation of TH metabolic processes.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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