Biochemical and Biophysical Research Communications 483 (2017) 541–546

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Follicular thyroglobulin induces cathepsin H expression and activity in thyrocytes

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ARTICLE INFO

Article history:
Received 30 November 2016
Accepted 16 December 2016
Available online 18 December 2016

Keywords:
Thyroglobulin
Cathepsin H
Thyroid hormone

ABSTRACT

Thyroglobulin (Tg) stored in thyroid follicles exerts a potent negative-feedback effect on each step of pre-hormone biosynthesis, including *Tg* gene transcription and iodine uptake and organification, by suppressing the expression of specific transcription factors that regulate these steps. Pre-hormones are stored in the follicular colloid before being reabsorbed. Following lysosomal proteolysis of its precursor, thyroid hormone (TH) is released from thyroid follicles. Although the suppressive effects of follicular Tg on each step of pre-hormone biosynthesis have been extensively characterized, whether follicular Tg accumulation also affects hormone reabsorption, proteolysis, and secretion is unclear. In this study we explored whether follicular Tg can regulate the expression and function of the lysosomal endopeptidases cathepsins. We found that in the rat thyroid cell line FRTL-5 follicular Tg induced cathepsin H mRNA and protein expression, as well as cathepsin H enzyme activity. Double immunofluorescence staining showed that Tg endocytosis promoted cathepsin H translocalization into lysosomes where it co-localized with internalized Tg. These results suggest that cathepsin H is an active participant in lysosome-mediated pre-hormone degradation, and that follicular Tg stimulates mobilization of pre-hormones by activating cathepsin H-associated proteolysis pathways.

1. Introduction

Thyroglobulin (Tg) is the most abundant protein produced by thyrocytes and it accumulates within thyroid follicles [1]. Newly synthesized Tg is iodinated and stored in the follicular lumen as a pre-hormone before being endocytosed back into the thyrocytes to yield the ultimate product, thyroid hormone (TH). Thyroid stimulating hormone (TSH) induces the endocytosis of follicular colloid into thyrocytes, which is followed by lysosomal fusion with endosomes where the pre-hormone is degraded to release T4, T3, and iodotyrosines [2]. However, the detailed mechanisms involved in this pre-hormone degradation are not fully understood.

A feedback effect of follicular Tg in the multi-step process of pre-hormone biosynthesis was recently demonstrated [3–10]. *In vitro* studies using rat thyroid FRTL-5 cells and *in vivo* studies with rat thyroids suggested that individual follicles were stimulated to synthesize pre-hormone (i.e., iodinated Tg) by TSH, but were also negatively regulated by Tg protein that accumulates in each follicle through concentration-dependent suppression of the expression of thyroid-specific functional genes at a transcriptional level [4–10]. This negative-feedback effect of follicular Tg was recently confirmed in primary cultures of normal human thyrocytes [11]. Based on these findings, a 'follicular cycle model' was proposed to describe the current understanding of how follicular function is regulated. In this model, Tg stored in the follicular lumen counteracts TSH actions to maintain a well-organized cycle of hormone

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http://dx.doi.org/10.1016/j.bbrc.2016.12.109
0006-291X/

synthesis and secretion from each follicle [3]. Thus, as sufficient amounts of Tg accumulate in a follicular lumen, the follicle would accordingly downregulate the rate of pre-hormone biosynthesis, which may be accompanied by accelerated rates of endocytosis to release and secrete TH into the circulation. In agreement with the predictions implied by this 'follicular cycle model', recent data showed that follicular Tg significantly and dose-dependently induces the expression of the TH transporter monocarboxylate transporter 8 (MCT8) in thyrocytes [12,13].

Several lysosomal endopeptidases are present in the thyroid, including cathepsins D, B, and H [14,15]. TSH was shown to stimulate cathepsin B and D expression to promote Tg proteolysis and hormone release by thyrocytes [14,16]. Inhibition of cathepsin enzymatic activity blocked Tg degradation, suggesting that these endopeptidases are important for Tg proteolysis [17]. In this study we explored the potential effect of follicular Tg on cathepsin expression and function.

2. Materials and methods

2.1. Cell culture and treatment

Rat thyroid FRTL-5 cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% bovine serum (Invitrogen, Waltham, MA) and a six-hormone mixture (1 mU/ml TSH, 10 mg/ml insulin, 10 ng/ml somatostatin, 0.36 ng/ml hydrocortisone, 5 mg/ml transferrin, and 2 ng/ml glycyl-L-histidyl-L-lysine acetate) as previously described [10,13,18]. Bovine Tg (Sigma Aldrich, St. Louis, MO) was dissolved in the culture medium and used at a final concentration of 5 mg/ml. Bovine serum albumin (BSA) (Sigma-Aldrich) at the same concentrations was used as a control.

2.2. Total RNA isolation and real-time PCR

Total RNA was purified using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as previously described [11]. Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) and the StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The sequences of PCR primers were: *Ctsh* forward, 5′-TCCTACGATGAGAATGCCGC-3′; *Ctsh* reverse, 5′-ACAAAGCGTATGGCTGGTGA-3′; *Ctsb* forward, 5′-AGGCTGGACGCAACTTCTAC-3′; *Ctsb* reverse 5′-CCAAATGCCCAACAAGAGCC-3′; *Ctsd* forward, 5′-CTATAAGCCGGCGACCTCTG-3′; *Ctsd* reverse, 5′-GCGCAGGGGGATTCTGATAA-3′; *Gapdh* forward, 5′-ACAGCAACAGGGTGGTGGAC-3′; and *Gapdh* reverse, 5′-TTTGAGGGTGCAGCGAACTT-3′. Relative mRNA expression levels were normalized against corresponding *Gapdh* levels. Real-time PCR analysis was carried out at least in triplicate, and repeated at least three times.

2.3. Protein preparation and Western blotting

Cells incubated with bovine Tg for 48 h were lysed in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 0.1% NP40, 20% glycerol, and a cOmplete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Basel, Switzerland) for 1 h at 4 °C. The lysates were centrifuged for 20 min at 4 °C to recover cellular proteins. Protein concentrations were determined using DC protein assay reagents (BIO-RAD, Hercules, CA) and a VMax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. Proteins were separated on NuPage 4–12% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes using Novex iBlot PVDF transfer stacks (Life Technologies, Waltham, MA) with an iBlot2 Gel Transfer Device (Life Technologies). The membranes were washed with Dulbecco's phosphate buffered saline (DPBS) (Sigma Aldrich) containing 0.1% Tween 20 (PBST), blocked with PBST containing 5% nonfat dry milk for 1 h, and then incubated overnight at 4 °C with a rabbit anti-cathepsin H antibody (ab128907, Abcam, Cambridge, UK; 1:500) or a goat anti-β-actin antibody (Santa Cruz Biotechnology, Dallas, TX; 1:1000). After washing with PBST, membranes were incubated with either donkey anti-rabbit IgG-biotin conjugates (GE Healthcare, Little Chalfont, UK; 1:20,000) or donkey anti-goat IgG-biotin conjugates (Millipore, Bellerica, MA; 1:20,000) for 1 h. Membranes were washed with PBST, and then incubated with streptavidin horseradish peroxidase (GE Healthcare; 1:20,000) for 1 h. Specific protein bands were visualized using Immunostar LD reagent (Wako Pure Chemical, Osaka, Japan) and captured with a C-DiGit blot scanner (LI-COR, Lincoln, NE) according to the manufacturer's instructions.

2.4. Cathepsin activity assay

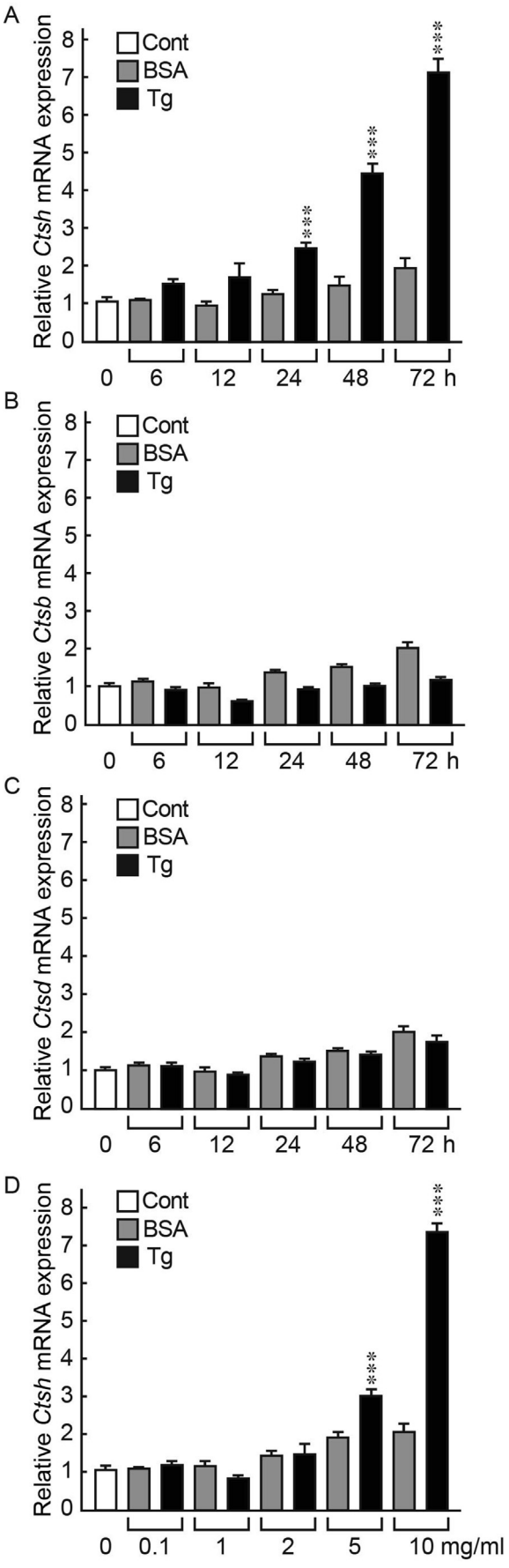
The activity of cathepsin H was evaluated using a Cathepsin H Activity Assay Kit (Abcam). Cellular proteins (10 μg) were mixed with assay buffers and arginine was labeled with amino-4-trifluoromethyl coumarin (AFC) as a substrate, before the mixture was pipetted into black Nunc MicroWell 96-well microplates (Life Technologies) according to the manufacturer's instructions. Specific fluorescence was measured using a F-7000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) according to the manufacturer's instructions.

2.5. Immunofluorescence staining

Cells were grown on poly-L-lysine coated culture coverslips (Matsunami Glass, Osaka, Japan) in a 24-well cell culture plate, and treated with 10 mg/ml Tg for 24 h or 48 h. To label lysosomes, LysoTracker® Red DND-99 (Life Technologies) was added to the culture medium at a final concentration of 50 nM and incubated with cells at 37 °C for 1 h. Cells were fixed with 10% buffered formalin (Wako Pure Chemical) for 15 min, permeabilized with 0.3% Triton X-100 (Wako Pure Chemical) in DPBS for 5 min, and blocked with 0.5% BSA in DPBS for 1 h. Immunofluorescence staining was performed by incubating the coverslips with a mouse anti-bovine Tg antibody (AM20511PU-N, Acris Antibody, San Diego, CA; 1:500) and/or a rabbit anti-cathepsin H antibody (Abcam; 1:200) overnight at 4 °C, then with an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Life Technologies; 1:1000) and an Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (Life Technologies; 1:1000) simultaneously, or with an Alexa Fluor 488-conjugated a chicken anti-rabbit IgG antibody (Life Technologies; 1:1000) for 1 h at room temperature. The nuclei were counterstained with Hoechst 33258 (Life Technologies; 1:1000) and the cover slips were secured with fluorescence mounting medium (Dako, Tokyo, Japan). Immunofluorescence was visualized and the images were captured with an FV10i-LIV laser scanning microscope (Olympus, Tokyo, Japan).

2.6. Statistical analysis

All experiments were repeated at least three times with different batches of cells, and the mean ± SD of these experiments was calculated. The significance of the differences between experimental values was determined by an unpaired two-tailed *t*-test, wherein $p < 0.05$ was significant.



3. Results

3.1. Follicular Tg induces mRNA and protein expression of cathepsin H in rat thyroid FRTL-5 cells

To explore the potential role of follicular Tg in the enzymatic degradation of iodinated Tg protein itself, we first examined the effect of follicular Tg on mRNA expression of cathepsin B, D, and H in rat thyroid FRTL-5 cells using real-time PCR. Cathepsin H (*Ctsh*) mRNA expression was significantly induced by 10 mg/ml Tg in a time-dependent manner up to 72 h, whereas BSA had no effect (Fig. 1A). However, Tg treatment did not affect either cathepsin B (*Ctsb*) or D (*Ctsd*) mRNA levels (Fig. 1B and C, respectively). Measurement of *Ctsh* mRNA expression at 72 h following treatment with increasing doses of Tg (Fig. 1D) suggested that follicular Tg would have a stimulatory effect on *Ctsh* gene expression when pre-hormone accumulates to high levels. In accordance with the mRNA expression levels, Western blotting showed that cathepsin H protein expression was specifically induced by Tg at higher concentrations (Fig. 2A) after 72 h (Fig. 2B). These results indicate that sufficient accumulation of Tg in the follicular lumen would induce cathepsin H expression in surrounding thyrocytes.

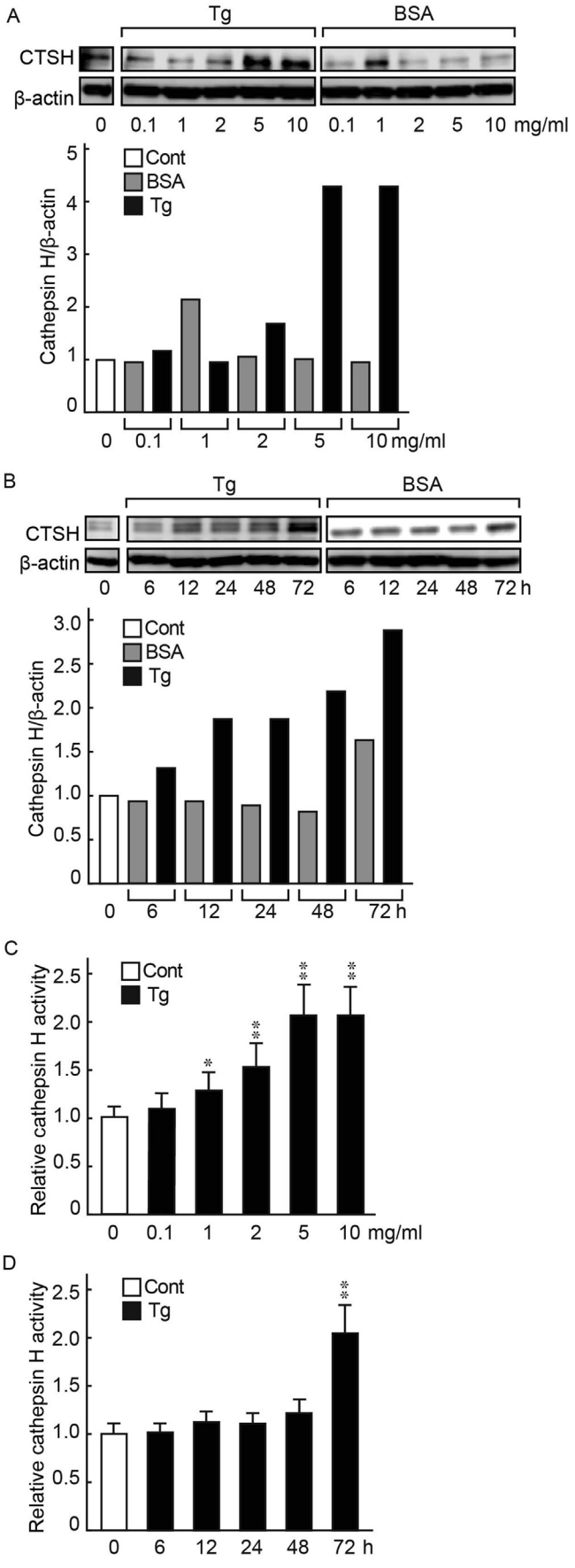
3.2. Follicular Tg increases enzymatic activity of cathepsin H in FRTL-5 cells

Cathepsins are synthesized as pre-proenzymes and acquire enzymatic activity after proteolytic processing within the lysosomes [19]. Therefore, we next tested the effect of follicular Tg on cathepsin H activity using the preferred cathepsin substrate amino-4-trifluoromethyl coumarin (AFC) labeled with arginine in a fluorometric assay. In this assay, active cathepsin H in cell lysates cleaves the synthetic substrate arginine-AFC to release free AFC, which has a fluorescence signal that can be quantified using a fluorescence plate reader. With this assay, we found that Tg treatment of FRTL-5 cells significantly and dose-dependently increased cathepsin H activity at 72 h (Fig. 2C, D). These results indicate that follicular Tg not only induces cathepsin H expression, but also enhances its enzymatic activity in thyrocytes.

3.3. Tg endocytosis induces lysosomal translocalization of cathepsin H

After synthesis in the endoplasmic reticulum (ER) and post-translational modification in the trans-Golgi network, cathepsins are packed into clathrin-coated vesicles and transported to late endosomes, which can then fuse with lysosomes where cathepsins are activated by low pH [20,21]. To demonstrate potential changes in the intracellular distribution of cathepsin H upon Tg endocytosis, we performed double immunofluorescence staining for cathepsin H and internalized Tg in FRTL-5 cells. Lysosomes, the organelles where cathepsins function, were simultaneously labeled with LysoTracker as described in Materials and methods. The results demonstrated that lysosomes were actively formed only after Tg was endocytosed and translocated to the lysosomes (Fig. 3A). Cathepsin H initially had an even cytoplasmic distribution in the

Fig. 1. Tg induces mRNA expression of *Ctsh*. FRTL-5 cells were treated with 10 mg/ml Tg or BSA for the indicated time period for up to 72 h (A–C) or treated with 0.1–10 mg/ml of Tg or BSA for 72 h (D). Total RNA was purified from the cells and subjected to real-time PCR analysis to determine the relative mRNA expression levels of *Ctsh* (A and D), *Ctsb* (B), and *Ctsd* (C). mRNA levels were normalized against *Gapdh* levels, and expressed as fold-change relative to the control. ***$p < 0.001$, compared to the control levels. Data are presented as mean ± SD relative to control levels (n = 3).



control cells, but translocalized in part to the lysosomes after Tg endocytosis (Fig. 3B), indicating that cathepsin H activation would occur after Tg endocytosis. Furthermore, in Tg-treated cells cathepsin H was indeed co-localized partially with the internalized Tg in vesicle-like structures (Fig. 3C), supporting the notion that cathepsin H is one active participant in the enzymatic degradation of pre-hormones in thyrocytes. These results together suggest that follicular Tg likely activates a cathepsin H-associated lysosomal proteolysis pathway that leads to pre-hormone mobilization in thyrocytes.

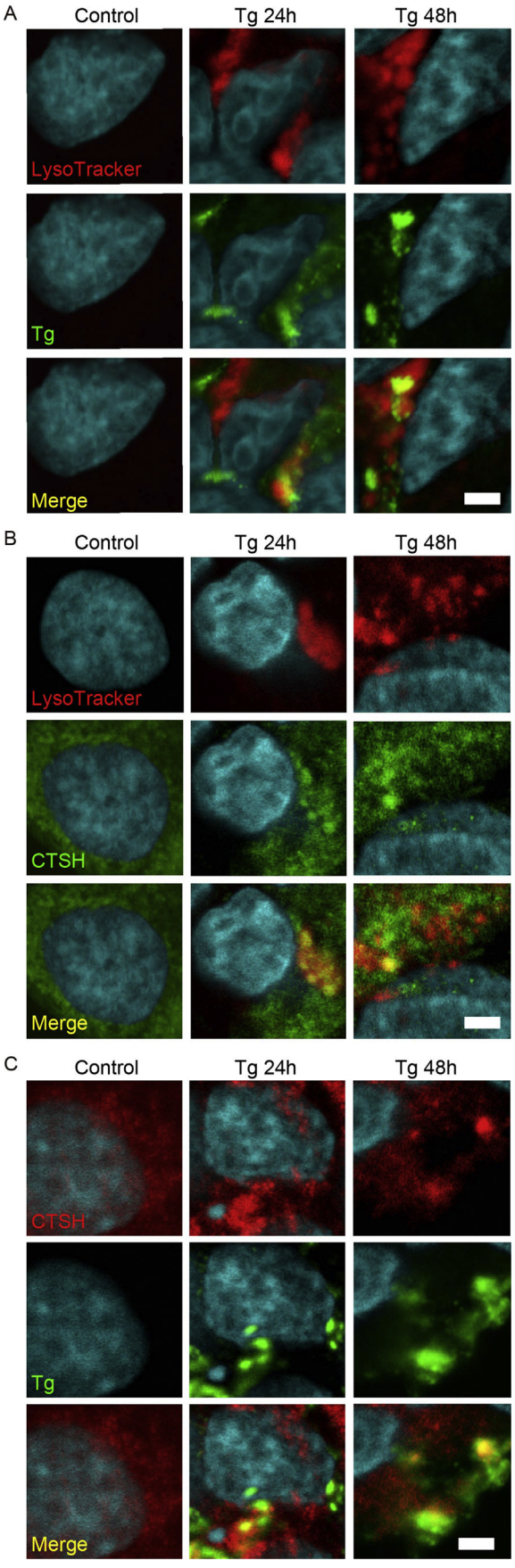
4. Discussion

In the current study, we demonstrated that follicular Tg induced mRNA and protein expression of cathepsin H, and also increased its enzymatic activity in rat thyroid FRTL-5 cells. Moreover, upon Tg stimulation, intracellular cathepsin H, which was initially distributed evenly in the cytoplasm, became concentrated in lysosomes where it co-localized with a fraction of the internalized Tg. These results together indicate that upon reaching sufficient concentrations within the follicular colloid, follicular Tg likely activates a cathepsin H-associated lysosomal degradation pathway that leads to hormone secretion from the follicle.

The thyroid gland is comprised of thyroid follicles, which are the minimal functional unit of the thyroid. Despite the seemingly uniform TSH stimulation among follicles, the morphology and function of each follicle can vary significantly. Thus, follicles in the same normal thyroid tissue can have a broad distribution of size, colloid Tg concentrations, iodide uptake, TH accumulation, enzymatic activities, and expression of thyroid-specific transcription factors [3]. This phenomenon, termed follicular heterogeneity, suggests that factors other than TSH levels in the blood are involved in regulating individual follicular function. We previously showed that follicular Tg exerts an intrinsic feedback regulatory effect on pre-hormone biosynthesis in a concentration-dependent manner [4–10].

The ‘follicular cycle model’ has been proposed to describe TH production in each follicle that is not only stimulated by TSH but also regulated by follicular Tg [3]. In a follicle with low amounts of accumulated Tg, pre-hormone synthesis is relieved from a negative-feedback effect of follicular Tg, but dominantly stimulated by TSH (i.e., the ‘synthesis phase’), which leads to an increased amount of accumulated follicular Tg. This accumulated follicular Tg in turn suppresses pre-hormone synthesis in a concentration-dependent manner, and eventually stops synthesis when Tg levels are high (i.e., the ‘storage phase’). Then, the follicles undergo pre-hormone mobilization through lysosomal degradation, and secrete TH (i.e., ‘secretion phase’). Thus, the accumulated pre-hormone would be consumed, which in turn relieves the negative-feedback effect of follicular Tg on pre-hormone biosynthesis to allow the follicle to re-enter the ‘synthesis phase’. As such, synthesis, storage, and secretion phases cycle at an intrinsic rate set by Tg accumulation in each follicle [3–9].

Fig. 2. Tg induces protein expression and enzyme activity of cathepsin H. FRTL-5 cells were treated with 0.1–10 mg/ml Tg or BSA for 72 h (A, C), or treated with 10 mg/ml Tg or BSA for the indicated time period up to 72 h (B, D). Whole cell proteins were extracted for subsequent analyses. Western blot analysis was performed to determine the protein expression levels of cathepsin H and β-actin. The densities of specific bands were determined using Image Studio Digits software, and the relative protein expression levels were normalized against β-actin and shown as fold-change relative to the control (A, B). Fluorometric assays was performed to determine cathepsin H enzyme activity. The data were normalized relative to corresponding protein concentrations. $^{*}p < 0.05$; $^{**}p < 0.01$, compared to the control. Data are presented as mean ± SD relative to control (n = 3) (C, D).



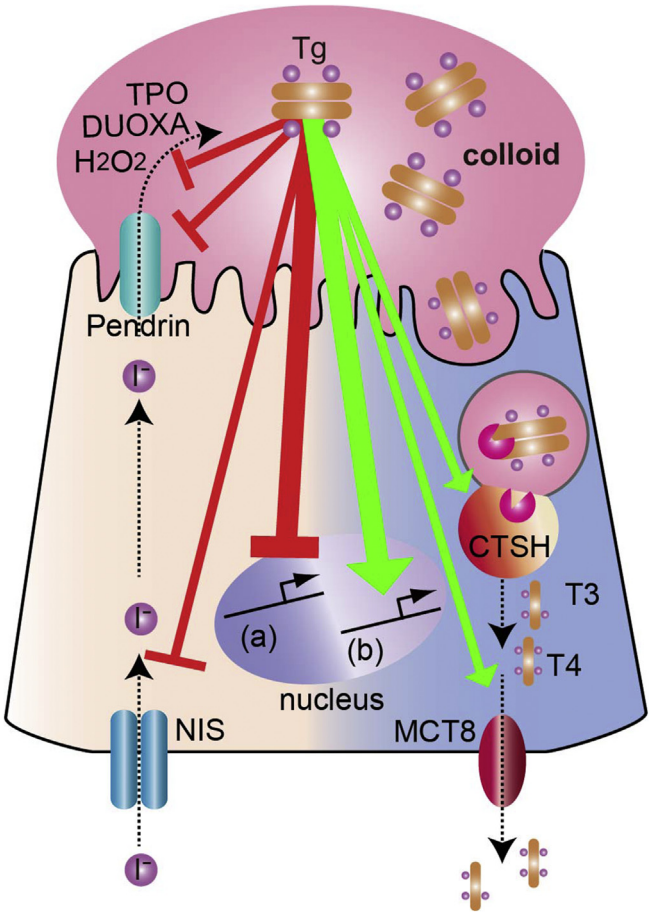


Fig. 4. Schematic representation of follicular Tg effects on pre-hormone biosynthesis, enzymatic proteolysis, and TH secretion in thyrocytes. Two opposing vectorial processes occur in thyrocytes (delineated by dotted lines): i) pre-hormone inflow towards the follicular lumen, which includes iodide uptake and transport, Tg protein expression and oranification; and ii) TH outflow towards circulation, which includes pre-hormone reabsorption, enzymatic proteolysis, and TH secretion. Tg accumulated in the follicular lumen inhibits pro-hormone inflow by suppressing expression of genes that are necessary for this process (a), and promotes TH outflow by inducing expression of relevant genes, including *CTSH* and *MCT8* (b).

This 'follicular cycle model' is strongly supported by several lines of evidence. In both rat and human thyrocytes, follicular Tg dose-dependently suppresses the expression of virtually all genes, including *Tg* itself, sodium iodide symporter (*Slc5a5*, an integral membrane protein that transports two Na^+ and one I^- simultaneously), thyroid peroxidase (*Tpo*), dual oxidase 2 (*Duox2*) and dual oxidase maturation factor 2 (*Duoxa2*), that are essential for the pre-hormone biosynthesis [4–11] (Fig. 4). Moreover, follicular Tg significantly induces the expression of the TH transporter MCT8 on the basal side of thyrocytes [13] (Fig. 4), which is consistent with a positive-feedback effect of follicular Tg on the 'secretion phase' that was predicted by the 'follicular cycle model'. In addition to TH secretion through MCT8, lysosomal pre-hormone degradation is

Fig. 3. Tg endocytosis induces lysosomal translocalization of cathepsin H in thyrocytes. FRTL-5 cells were treated with 10 mg/ml Tg for 24 or 48 h. Lysosomes were labeled with LysoTracker (red) and the internalized Tg was labeled with immunofluorescence (green) (A). Lysosomes were labeled with LysoTracker (red) while cathepsin H (CTSH) was labeled with immunofluorescence (green) (B). Double immunofluorescence staining was performed for CTSH (red) and internalized Tg (green) (C). Nuclei were counterstained with Hoechst 33258 (blue). Bars: 5 μm.

obviously another essential step to yield TH in follicles. The effect of follicular Tg on this particular step was addressed by this study (Fig. 4). Here we demonstrated that follicular Tg significantly induced both expression and activity of the lysosome proteinase cathepsin H, which also supports the prediction that accumulated Tg protein in each follicle promotes the 'secretion phase' that likely embodies pre-hormone mobilization through a cathepsin H-associated lysosomal degradation pathway.

Cathepsins B, D, and H have been reported to be expressed in thyrocytes, and are involved in the lysosomal degradation of pre-hormones [14,17]. Follicular Tg specifically induces the gene expression of cathepsin H, but not that of cathepsin B and D, implying that cathepsin H may play a key role in Tg degradation. The feedback effect of follicular Tg on thyroid-specific functional genes acts at a transcriptional level by modulating the expression and binding of thyroid specific transcription factors [5,7,22]. The present study showed that follicular Tg induced gene expression of cathepsin H, and also enhanced its enzymatic activity in thyrocytes. We also showed that Tg promotes translocalization of cathepsin H to lysosomes where it can interact with lysosome-internalized Tg. These results are in agreement with a predicted positive role for follicular Tg in the 'secretion phase' that was suggested in the 'follicular cycle model'. The current finding of Tg-dependent regulation of cathepsin H gene expression and activity provides invaluable insight to better understand how Tg affects hormone absorption, proteolysis, and secretion in thyroid follicles.

Conflict of interests

No conflict of interests were stated by authors.

Acknowledgements

This work was supported in part by a JSPS KAKENHI Grant (#15K09444 to K.S.).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.12.109.

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