TSH regulates a gene expression encoding ERp29, an endoplasmic reticulum stress protein, in the thyrocytes of FRTL-5 cells

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Abstract This experiment was performed to evaluate the effect of thyroid-stimulating hormone (TSH) on the endoplasmic reticulum resident 29 kDa protein (ERp29) gene expression in the thyrocytes of FRTL-5 cells. Although ERp29 mRNA was constantly expressed, its expression began to increase remarkably from 10^{-9} M TSH. On the other hand, the effect of TSH on the abundance of ERp29 mRNA started within 6 h and peaked at 8 h. Actinomycin D strongly blocked this effect while cycloheximide did not. The half-life of ERp29 mRNA was about 4–4.5 h in the presence or absence of TSH that was not affected by the stability of ERp29 mRNA. The effect of TSH on the ERp29 gene expression was specific, while other growth factors (transferrin, insulin and hydrocortisone) did not alter its expression. Our data indicate for the first time that the expression of ERp29 is regulated transcriptionally by TSH in thyrocytes.

Key words: Thyroid-stimulating hormone; FRTL-5 cell; Endoplasmic reticulum; Molecular chaperone

1. Introduction

One of the most important jobs of the endoplasmic reticulum (ER) is to provide an environment that facilitates the proper folding and assembly of newly synthesized secretory proteins and plasma membrane proteins. This organelle contains numerous ER chaperones, ER foldases and ER degradases that assist in ER quality control, including the degradation of mis/unfolded proteins that permit the exit of completely folded proteins only [1]. Recently, it has been reported that a new ER resident 29 kDa protein (ERp29) from the enamel cells is one of the highly conserved members of the ER chaperones (such as grp94, calreticulin, PDI and BiP). The ERp29 gene was found to be ubiquitously expressed and was activated in response to ER stress. The cDNA of ERp29 encodes a putative 260 amino acids with 28 574 Da and p*I* 5.6. The ERp29 protein was abundant (approximately 0.2% of soluble protein) and had an ER-retrieval signal, the C-terminal tetrapeptide KEEL. In the cells, the ERp29 protein interacts with an ER chaperone of BiP/grp78 and is assembled in a homodimer with a thioredoxin-like structure [2–4].

Most ER resident stress proteins are induced transcriptionally in response to the starvation of glucose, glycosylation inhibition, perturbation of ER stores of Ca^{2+}, blocking the transport of proteins from the ER to the Golgi body and induction of reducing agents and heavy metals [5–9]. Although some information for the ERp29 gene itself and the proteins widely expressed in animal tissues and stress-inducible in the ER were reported [2–4], the exact biological function of ERp29 and its gene expression by hormone are still unclear. With this in mind, we tested the hormonal expression of ERp29 in the cells whose expression was thyroid-stimulating hormone (TSH)-dependently increased, and chose the thyroid culture cells (FRTL-5 cells) that have been well studied with respect to ER chaperones [10,11].

As a step toward elucidating the function of the ERp29 in thyrocytes, it was necessary to know how the ERp29 gene is controlled by TSH in FRTL-5 cells. Actually, it is well known that TSH dominantly regulates the thyroid hormone synthesis in the thyrocytes [12]. We recently isolated the cDNA fragment that encodes partial ERp29 from the FRTL-5 cells using a differential display-PCR method, which was TSH-dependently regulated under the transcription level [13]. At present, however, the effect of TSH on the ERp29 gene expression is still quite unclear. With this in mind, we investigated the mRNA expression of ERp29 in the cellular response against TSH by Northern blot analysis. The data in this study cover the first set of results, showing that TSH specifically controls ERp29 gene expression in the thyrocytes.

2. Materials and methods

2.1. Materials

Highly purified bovine TSH was obtained either from the hormone distribution program of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health (NIDDK-bTSH I-1: 30 U/mg) or was a previously described preparation, 26 ± 3 U/mg, homogeneous in the ultracentrifuge, about 27 500 in molecular weight, with the amino acid and carbohydrate composition of TSH. [^{32}P]dATP (3000 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA, USA) [14]. The source of all other materials used in this study was Sigma (St. Louis, MO, USA) unless otherwise noted.

2.2. Cell culture

The rat thyroid cell line FRTL-5 (American Type Culture Collection, CRL #8305, Rockville, MD, USA) was cultured in Coon's medium, containing 5% calf serum and 10^{-9} M TSH, 5 μg/ml transferrin, 1 μg/ml insulin and 10 nM hydrocortisone, at 37°C in a humid atmosphere with 5% CO_2 [10]. Their doubling time with TSH was 36 ± 6 h; they did not proliferate without TSH. The cells were diploid

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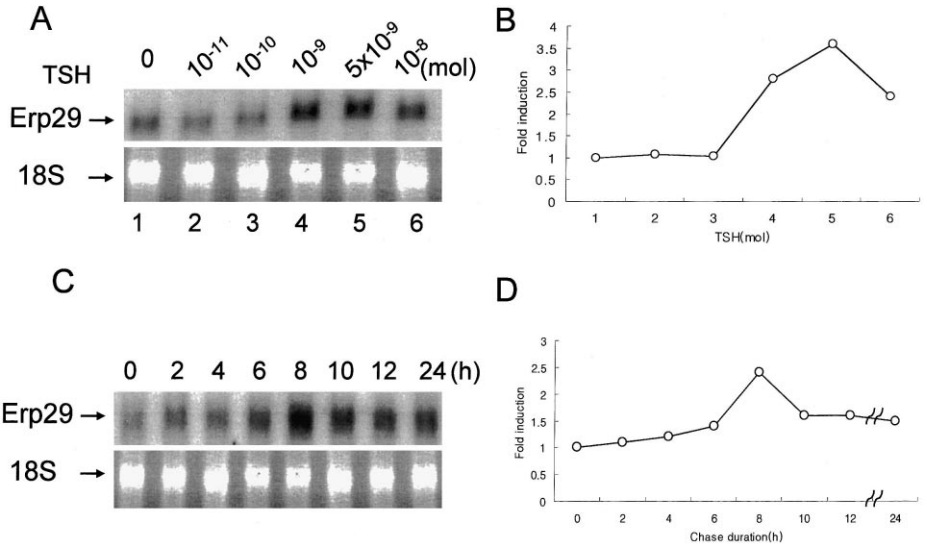

Fig. 1. TSH influences ERp29 mRNA expression: dose- and time-dependent induction. Confluent FRTL-5 cells were treated with the indicated doses of TSH at 37°C for 24 h, after treatment for 2 days with 5% calf serum and without 4H in the growth medium. Total RNA was isolated as described in Section 2 and was subjected to Northern blotting. A: TSH enhancement of the ERp29 mRNA. An arrow indicates ERp29 (upper panel), and an ethidium bromide staining of the 18S ribosomal RNA (lower panel) was used to indicate the equivalence of the load on the gel. B: Quantification of ERp29 mRNA bands in (A). C: The effects of Erp29 on TSH mRNA over time. Each cell treated with 10^{-9} M TSH for 24 h in the 4H medium. An arrow indicates ERp29 (upper panel) and an ethidium bromide staining of the 18S ribosomal RNA (lower panel) was used to indicate the equivalence of the load on the gel. D: The quantification of (C) by phosphorimaging. Both (B) and (D) were performed with duplicate samples and the results were averages of repeated experiments. Statistical comparison of paired mean values (i.e. TSH treatment versus control).

between their 5th and 20th passage. A fresh medium was added to the cells every 2 or 3 days and the cells were passaged every 2 weeks. As an individual experiment, the cells were washed three times with cold phosphate-buffered saline (PBS) to completely remove the hormones and then were incubated for 48 h in growth medium without hormones.

2.3. RNA isolation and Northern analysis

After various TSH concentrations, incubation times and treatment of other agents, the confluent FRTL-5 cells were scraped from the Petri dishes with a rubber policeman into 10 ml of cold PBS and collected by clinical centrifugation for 3 min at 2000×*g*. The pellet was disrupted by Dounce homogenization with a tight-fitting pestle in 15 ml of lysis buffer (10 mM Tris, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 0.25% Triton X-100). The suspension containing total RNA was layered on top of a 1.2 ml cushion of 5.7 M CsCl in 0.1 M EDTA and centrifuged at 15°C for 24 h at 36 000 rpm in a Beckman SW55 rotor. The RNA pellet was treated with 500 μg/ml of proteinase K in an extraction buffer (1% SDS, 0.01 mM Tris, pH 7.5, 5 μM EDTA). After phenol and ether extractions, the RNA pellet was suspended in DEPC-treated water and the yield was determined twice for each sample using an UV spectrophotometer.

The mRNA expression level of the ERp29 was analyzed by Northern blotting. Equal amounts of RNA (20 μg) were separated on a denaturing agarose gel (2.5% agarose containing 2.2 M formaldehyde) and transferred to a nylon membrane (Boehringer, Germany). After the filter was UV-crosslinked (UV-Stratalinker 1800, Stratagene), it was prehybridized in a high SDS buffer (7% SDS, 50% formamide, 5×SSC, 2% blocking reagent, 50 mM sodium phosphate) at 50°C for 2 h. Hybridization was performed with the same SDS buffer containing [^{32}P]dATP leveled ERp29 DNA fragments at 50°C overnight. The nylon membrane was rinsed twice with 2×SSC and 0.1% SDS at room temperature. The membrane was then exposed to an X-ray film in a deep-freeze for a period of time between 5 h and 1 day to allow the mRNA signals to develop. The ERp29 DNA fragments were isolated from the agarose gel by a silica-based matrix of DNA PreMate™ Kit (Bioneer, Korea) and were radio-labeled by the random primer method using a kit (Amersham Life Science, Arlington Heights, IL, USA) and used as molecular probes for Northern blot analysis. For quantification, a phosphorimaging analyzer was used to measure the hybridization signals (Molecular Dynamics).

3. Results and discussion

3.1. TSH dose/time-dependent ERp29 mRNA expression

We first determined whether the ERp29 gene was activated in the thyrocytes of the FRTL-5 cells stimulated by TSH. The cells were incubated in a control growth medium with 5% calf serum and without TSH, transferrin, insulin or hydrocortisone (called 4H) for 2 days to completely remove the hormonal effect. Various concentrations of TSH were added to the growth media for 24 h. As shown in Fig. 1A,B, the ERp29 mRNA was constantly expressed in the absence of TSH (control) and its expression began to increase gradually relative to the added TSH concentration.

When 10^{-9} M TSH was added, a remarkable expression was detected in the FRTL-5 cells. No data for the gene expression mechanism of ERp29 by TSH have ever been shown, so this finding is the first to show that TSH increased the mRNA level of ERp29 in the thyrocytes. Considering the fact that ERp29 belongs to the ER stress protein, similar expression patterns are not a strong argument because, in general, the heat shock protein expression increased rapidly only early on and for a short time after external stimulations (e.g. heat, heavy metal, metabolically toxic matters or unfolded protein accumulation) [5–9]. Based on the results shown in Fig. 1, we suggest that ERp29 mRNA expression is required for the physiological role under normal cell conditions and TSH enhances its expression.

Next, we examined the effect of TSH on the ERp29 mRNA expression over time during a 24 h exposure. The FRTL-5 cells were incubated for the indicated number of hours in the presence of 10^{-9} M TSH after preincubation for 2 days in a growth medium with 5% calf serum and without 4H (Fig. 1C,D). Although the signal of the ERp29 expression was detected when the cells were not chased, its expression was in-

creased gradually by chase periods for 24 h. There was a time-dependent increase in the ERp29 mRNA expression, which peaked at 8 h (approximately five-fold) in three independent experiments compared to its measure at 0 h, and then decreased thereafter.

Recent studies have revealed the early expression of genes in the thyrocytes in response to growth, proliferation and differentiation stimuli, but they did not describe the stress-inducible genes. The mRNA levels of the tested genes (c-*myc*, *jun* B and *fos* B) were strongly increased, but the mRNA levels of c-*jun* and *egr* were decreased by TSH. Those remarkable expression signals were detectable within 2–4 h after the TSH-treatment [15]. The ERp29 gene shows immediate early expression due to stress, but at the present we do not fully understand its expression mechanism. It is not so strange that the ERp29 gene expression (by TSH) takes longer (approximately 6–8 h as shown in Fig. 1C,D) compared to stresses.

3.2. Effects of transcription and protein synthesis inhibitors for the ERp29 expression

To investigate the mode of TSH action in the regulation of the ERp29 gene expression, the FRTL-5 cells were treated for 24 h with actinomycin D (AD; inhibitor for transcription) [16] and cycloheximide (CH; inhibitor for protein synthesis) [17] in the presence or absence of 10^{-9} M TSH after preincubation for 2 days in the growth media with 5% calf serum and without 4H. The result of Northern blotting is shown in Fig. 2. As already shown in Fig. 1, TSH alone fully increased the ERp29 mRNA expression in the FRTL-5 cells. When the cells were treated with 0.2 μg/ml AD in the presence or absence of TSH, the resulting signal of ERp29 mRNA was completely abolished whether TSH was present or not.

On the other hand, for the cells treated with 2 μg/ml CH in the presence or absence of TSH, the expression pattern of ERp29 was similar to that of the control, although its expression was weak. This weakness is due to the activation of preexisting ERp29 mRNAs, which already become aggravated before the AD treatment. This result suggests that the ERp29 mRNA expression is completely regulated at the transcriptional level but there is no translational/posttranscriptional regulation in the thyrocytes. Although TSH alone fully enhances the ERp29 expression, TSH promotes only one expression pathway for the transcriptional up/down regulation of the

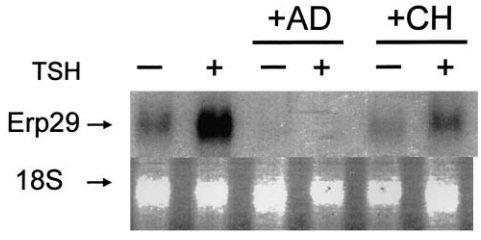

Fig. 2. Effects of AD and CH on TSH-induced accumulation of ERp29 mRNA. Confluent FRTL-5 cells were treated either with (+) or without (−) 10^{-9} M TSH for 24 h in the presence or absence of 0.2 μg/ml AD or 2 μg/ml CH after 48 h without any hormones as the growth medium. Total RNA was isolated as described in Section 2 and was subjected to Northern blotting. An arrow indicates ERp29 (upper panel) and an ethidium bromide staining of the 18S ribosomal RNA (lower panel) was used to indicate the equivalence of the load on the gel.

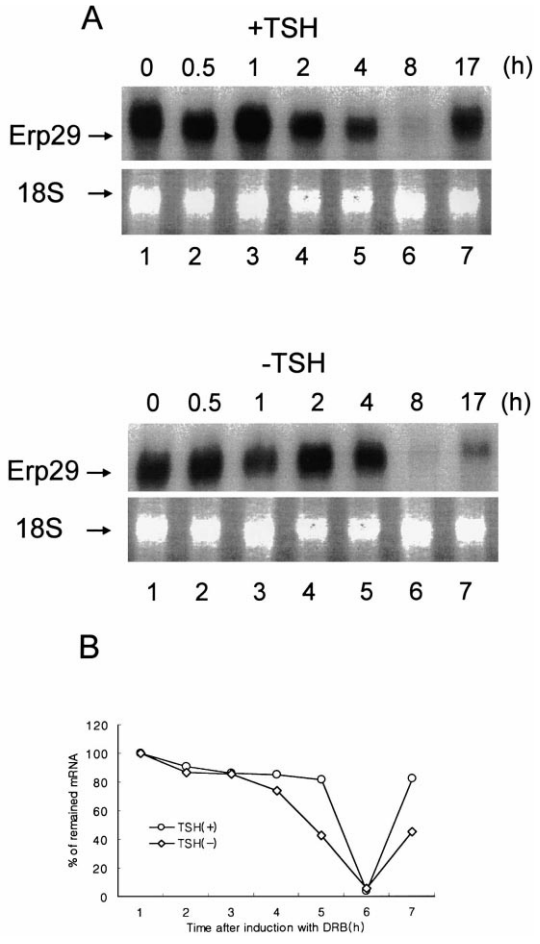

Fig. 3. Effect of TSH on the stability of ERp29 mRNA in FRTL-5 cells. A: Confluent FRTL-5 cells were treated with DRB (25 μg/ml) at 37°C and chased for 17 h in the presence (+) or absence (−) of 10^{-9} M TSH. Total RNA was isolated and subjected to Northern blotting as described in Section 2. An arrow indicates ERp29 (upper panels) and ethidium bromide staining of the 18S ribosomal RNA (lower panels) was used to indicate the equivalence of the load on the gel. B: Quantification of ERp29 mRNA bands in (A). The experiments above were performed with duplicate samples and the results were averages of repeated experiments. Statistical comparison of paired mean values (i.e. DRB treatment versus control). Presence (circles) or absence (rhombs) of 10^{-9} M TSH.

ERp29, as shown in Fig. 2. The cells treated with TSH were combined with AD, where the unenhanced signal was shown. If TSH had taken one of the alternative pathways for the ERp29 expression, some strong signal should have been shown when the cells were treated with TSH+AD.

3.3. Estimation of ERp29 mRNA half-life

We demonstrated the effects of TSH on ERp29 mRNA stability using 25 μg/ml DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), which is a specific inhibitor of RNA polymerase II [18]. The DRB treatment prevents new mRNA synthesis and permits the monitoring of intracellular residue mRNAs. As shown in Fig. 3A,B, no signals in either the presence or absence of TSH were commonly detected after 8 h of treatment of DRB on the FRTL-5 cells. This means that the already aggravated ERp29 mRNA was almost decayed, as the amount of ERp29 mRNA became aggravated just before the treatment of DRB. The half-life of ERp29

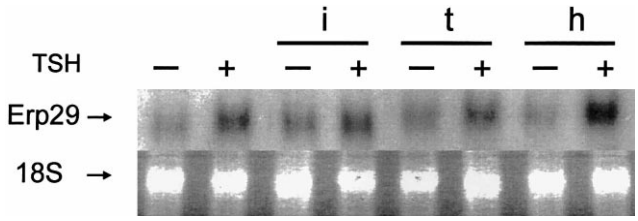

Fig. 4. Specificity of the TSH effect on the enhancement of ERp29 mRNA. Confluent FRTL-5 cells were treated for 24 h either with (+) or without (−) 10^{-9} M TSH in the presence of transferrin (5 μg/ml), insulin (1 μg/ml) and 10 nM hydrocortisone after 48 h without any hormones as the growth medium. Total RNA was isolated as described in and was subjected to Northern blotting. An arrow indicates ERp29 (upper panel), and an ethidium bromide staining of the 18S ribosomal RNA (lower panel) was used to indicate the equivalence of the load on the gel. Abbreviations: i (insulin), t (transferrin), h (hydrocortisone).

mRNA in the FRTL-5 cells was about 4 h in the absence of TSH and approximately 4.5 h in the presence of TSH.

Graphing the data from the DRB experiment, there was no serious difference between the control and TSH-treated groups. This means that the stability of the ERp29 mRNA was not largely affected in either the presence or absence of TSH in the FRTL-5 cells. That is to say, TSH is not involved in the step after transcription, which is effective only in the gene expression step. In addition, both signals seemed to be enhanced after 17 h of the DRB treatment because of the inactivation of the DRB that was already added by a single term of incubation (at 37°C) and because the signal when TSH was present appeared stronger than when TSH was absent.

3.4. TSH specific expression of ERp29

In general, the transcription of a gene requires several factors that make a large transcriptional complex [19]. Therefore, we examined the effects of these growth factors (e.g. transferrin, insulin, hydrocortisone; essential for growth of FRTL-5 culture cells) on ERp29 gene regulation in the presence or absence of TSH. The cells were treated for 24 h with 5 μg/ml transferrin, 1 μg/ml insulin and 10 nM hydrocortisone, in the presence or absence of 10^{-9} M TSH after preincubation for two days in the growth media with 5% calf serum and without 4H.

As shown in Fig. 4, the transferrin, insulin and hydrocortisone did not remarkably affect the constant ERp29 mRNA levels in the FRTL-5 cells in either the presence or absence of TSH. When TSH was added together with growth factors, the ERp29 expression was not additive and TSH alone increased the ERp29 mRNA levels. This shows that TSH specifically affects the ERp29 expression, at least in FRTL-5 cells, while the growth factors of transferrin, insulin and hydrocortisone play an important role in the growth of FRTL-5 cells. If the growth factors affect the ERp29 expression, some strong signals should have been shown when the cells were treated with a combination of TSH, transferrin, insulin and hydrocortisone. However, as shown in Fig. 4, no strong signals were found when the cells were treated with additional growth factors, compared to the signal produced by the treatment of TSH on its own.

In conclusion, our data demonstrate that TSH strictly controls the ERp29 gene expression at the transcriptional level in the thyrocytes. Considering that the ERp29 mRNA was constantly expressed in the cells that were not treated with TSH, in conjunction with the data from previous reports, it may be concluded that the ERp29 molecule is involved in the cell homeostatics (e.g. the ER-retrieval signal, stress inducibility, protein maturation in the ER and stress defense in the ER), as well as the association with other ER chaperones. Also, the TSH-specific expression of ERp29 suggests that it might have a unique role. We are now interested in the functional role of ERp29 as an ER chaperone in the thyrocytes, in that it might help the folding and assembly of the thyroid prohormone thyroglobulin.

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References

[1] Gething, M.-J. and Sambrook, J.F. (1992) Nature 355, 33–45.
[2] Demmer, J., Zhou, C.M. and Hubbard, M.J. (1997) FEBS Lett. 402, 145–150.
[3] Mkrtchiana, S., Baryshev, M., Matvijenko, O., Sharipo, A., Sandalova, T., Schneider, G. and Ingelman-Sundberg, M. (1998) FEBS Lett. 431, 322–326.
[4] Mkrtchiana, S., Fang, C., Hellman, U. and Ingelman-Sundberg, M. (1998) Eur. J. Biochem. 251, 304–313.
[5] Resendez, E., Attenello, J.W., Grafsky, A., Chang, C.S. and Lee, A.S. (1985) Mol. Cell. Biol. 5, 1212–1219.
[6] Li, W.W., Alexandre, S., Cao, X. and Lee, A.S. (1993) J. Biol. Chem. 268, 12003–12009.
[7] Kwon, O.-Y., Kim, Y.-J., Choi, Y., Kim, H., Song, C. and Shong, M. (1999) Z. Naturforsch. 54C, 573–577.
[8] Elbein, A.D. (1991) FASEB J. 5, 3055–3063.
[9] Lodish, H.F. and Kong, N. (1993) J. Biol. Chem. 268, 20598–20605.
[10] Ambesi-Impiombato, F.S., Parks, L.A. and Coon, H.G. (1980) Proc. Natl. Acad. Sci. USA 77, 3455–3459.
[11] Kuznetsov, G., Chen, L.B. and Nigam, S.K. (1994) J. Biol. Chem. 269, 22990–22995.
[12] Kim, P.S. and Arvan, P. (1993) J. Biol. Chem. 268, 4873–4879.
[13] Shong, M., Kim, Y.-J., Choi, Y. and Kwon, O.-Y. (1999) Z. Naturforsch. 54C, 578–582.
[14] Park, E.S., You, S.H., Kim, H., Kwon, O.-Y., Ro, H.K., Cho, B.Y., Taniguchi, S., Kohn, L.D. and Shong, M. (1999) Thyroid 9, 601–612.
[15] Deleu, S., Pirson, I., Clermont, F., Nakamura, T., Dumont, J.E. and Maenhaut, C. (1999) J. Cell Physiol. 181, 342–354.
[16] Inbal, N., Listovsky, T. and Brandeis, M. (1999) FEBS Lett. 463, 350–354.
[17] Minta, J. and Fung, M. (1999) Mol. Cell Biochem. 201, 111–123.
[18] Primorac, D., Johnson, C.V., Lawrence, J.B., McKinstry, M.B., Stover, M.L., Schanfield, M.S., Andjelinovic, S., Tadic, T. and Rowe, D.W. (1999) Croat. Med. J. 40, 528–532.
[19] Wolberger, C. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 29–56.