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Increase in Ref-1 mRNA and Protein by Thyrotropin in Rat Thyroid FRTL-5 Cells

Toshi Asai, Fukushi Kambe, Toyone Kikumori, and Hisao Seo
Department of Endocrinology and Metabolism, Division of Molecular and Cellular Adaptation, Research Institute of Environmental Medicine, Nagoya University, Nagoya 464-01, Japan

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Thyrotropin (TSH) induces the expression of *fos* and *jun* family genes in thyroid cells. The DNA-binding activity of these gene products (AP-1) has been shown to be enhanced by ubiquitous nuclear redox factor-1 (Ref-1). We thus examined whether TSH regulates Ref-1 gene expression in rat thyroid FRTL-5 cells. Northern blot analysis revealed that the abundance of Ref-1 mRNA significantly increased within 3 hours after TSH followed by a sustained increase until 12 hours. The increase was also induced by treatment with forskolin but not in the presence of cycloheximide, indicating that the TSH effect on Ref-1 mRNA is mediated by intracellular cAMP and requires *de novo* protein synthesis. Consistent with the elevation of the mRNA level, Western blot analysis showed an increase in Ref-1 protein 3 hours after TSH. The level continued to increase until 12 hours. These results suggested that increased Ref-1 by TSH might regulate the binding activity of AP-1 in thyroid cells. Considering that Ref-1 also has a DNA repair function, Ref-1 may play dual roles in gene regulation and DNA repair processes in thyroid cells.

Thyrotropin (TSH) induces the expression of *c-fos* and *c-jun* genes in thyroid follicular cells (1-4). Our previous study has demonstrated that TSH increases the expression of not only *c-fos* and *c-jun* genes but also other *fos* and *jun* family genes such as *fra-1, fra-2, fosB* and *junB* with a differential time course (5). The activator protein-1 (AP-1) consists of homodimer or heterodimer among *fos* and *jun* family gene products (6). It binds to an AP-1 site in a regulatory region of many vertebrate genes and regulates their expression (7).

The DNA-binding activity of AP-1 is regulated by a posttranslational mechanism involving redox (8). Redox regulation occurs through a conserved cysteine residue located in the DNA-binding domain of AP-1 (8). A ubiquitous nuclear redox factor-1 (Ref-1), also known as APEX nuclease (9), increases the DNA-binding activity by reducing the conserved cysteine (10, 11). It has been reported that Ref-1 is a major reductant for AP-1 in HeLa cells (11), because AP-1 binding activity was extremely decreased when Ref-1 protein in the cell extract was depleted by immunoprecipitation using specific antibody. Recently, Yao *et al* showed that Ref-1 and AP-1 were simultaneously increased in the human HT29 colon cancer cells when they were exposed to hypoxia (12). We thus examined whether Ref-1 gene expression is regulated by TSH or not, using a differentiated rat thyroid cell line FRTL-5.

MATERIALS AND METHODS

Cell culture. FRTL-5 cells (ATCC CRL8305) were cultured in Coon's modified Ham's F-12 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 5% heat-inactivated calf serum (GIBCO-BRL) and six hormones including hydrocortisone (10 nM), transferrin (5 μg/ml), somatostatin (10 ng/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), bovine TSH (1 mU/ml) and insulin (10 μg/ml) (13). After the cells reached to near confluency, the medium was replaced with that containing no TSH, no insulin and 0.5 % serum (basal medium). The cells were cultured in the medium for 4 days. The cells were then incubated with 1 mU/ml TSH or 10 μM forskolin for various length of time and harvested for Northern blot and Western blot analyses. In an experiment using cycloheximide (CHX, Wako, Tokyo, Japan), it was added to the media at a concentration of 10 μg/ml 15 min before TSH treatment.

Northern blot analysis. Total RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform-extraction method (14). The detailed procedures for Northern blotting were described in our previous report (15). After fractionation of 15 μg of total RNA on 0.8 % agarose gel, the gel was stained with ethidium bromide. The RNA was then transferred onto GeneScreen Plus membrane (Du Pont-New England Nuclear, Boston, MA). The amounts of 28S and 18S ribosomal RNAs in each lane were estimated from the photograph of the membrane taken under ultraviolet light (wave length: 320 nm). The membrane was hybridized with a rat Ref-1 cDNA probe labeled with [α-^{32}P]dCTP (New England Nuclear) using random primed labeling kit (Boehringer Mannheim, Germany). The probe contains a full coding sequence of rat Ref-1 (APEX nuclease) cDNA which was a gift from Dr. Seki, Okayama University Medical School, Okayama, Japan. After hybridization, the membrane was washed and exposed to XAR-5 film (Eastman Kodak, Rochester, NY) at −80 C with an intensifying screen. Quantitative analysis of the density

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of the specific bands were performed using BAS 2000 bioimage analyzing system (Fuji film co., Japan).

Western blot analysis. Whole cell extracts were prepared from FRTL-5 cells for Western blot analysis as described in our previous report (16). The extracts containing 60 μg of protein and Molecular Weight Standards (Bio-Rad, Richmond, CA) were fractionated on a sodium dodecyl sulfate (SDS)-12.5 % polyacrylamide gel, and electroblotted onto a membrane (Hybond-C super; Amersham Life Science, Arlington, IL) at 300 mA (2.5 mA/cm^2 gel) for 30 min using Mill Blot-SDE system (Millipore Corp., Bedford, MA). The part of molecular weight marker-blotted membrane was stained with Coomassie Brilliant Blue R (Sigma Chemical Co., St. Louis, MO). The remaining membrane was soaked for 30 min in a blocking buffer [TBST solution (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05 % Tween-20) containing 3% bovine serum albumin]. Then, the membrane was incubated for 30 min with anti-rat-Ref-1 rabbit serum (C-20; Santa Cruz Biotechnology, Inc. Santa Cruz, CA) diluted at 1: 500 with TBST. After washing three times with TBST, the membrane was incubated for 30 min with anti-rabbit-IgG goat IgG conjugated with alkaline phosphatase (Zeimed, San Francisco, CA) diluted at 1: 2000 with TBST. After washing three times, it was incubated in a color development solution [10 ml of AP buffer (100 mM Tris-HCl pH 9.5, 100mM NaCl, 5 mM $MgCl_2$) containing 66 μl of 50 mg/ml NBT (nitro blue tetrazolium in 70 % dimethylformamide) and 33 μl of 50 mg/ml BCIP (5-bromo-4-chloro-3-indolyl-phosphate in dimethylformamide)]. The color development was stopped by the incubation of the membrane in a stop solution (20 mM Tris-HCl pH 8.0, 5 mM EDTA).

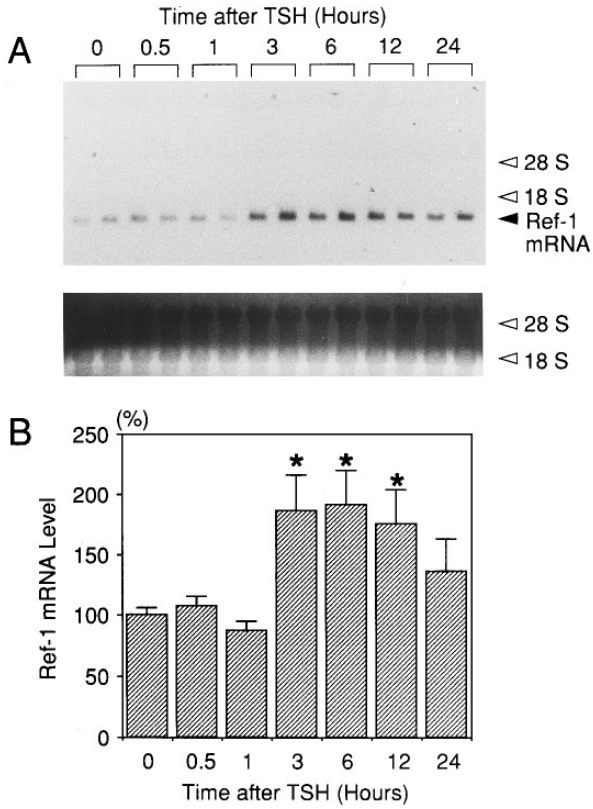

FIG. 1. TSH increases Ref-1 mRNA in FRTL-5 cells. FRTL-5 cells maintained in the basal medium for 4 days were exposed to 1 mU/ml TSH for various lengths of time and then harvested for Northern blot analysis. The experiments were performed in quadruplicate. A representative autoradiograph is shown in A. The positions of Ref-1 mRNA and of 28S and 18S ribosomal RNAs are indicated. The photograph of the membrane taken under ultraviolet light (wave length: 320 nm) depicting 28S and 18S ribosomal RNAs is shown in the lower panel. Changes in the amounts of Ref-1 mRNA by TSH were assessed by a BAS 2000 bioimage analyzing system and are depicted in B. Values are expressed as percentage of the value before TSH treatment (time 0) and the means ± SE are indicated. * $p < 0.05$ (vs. time 0).

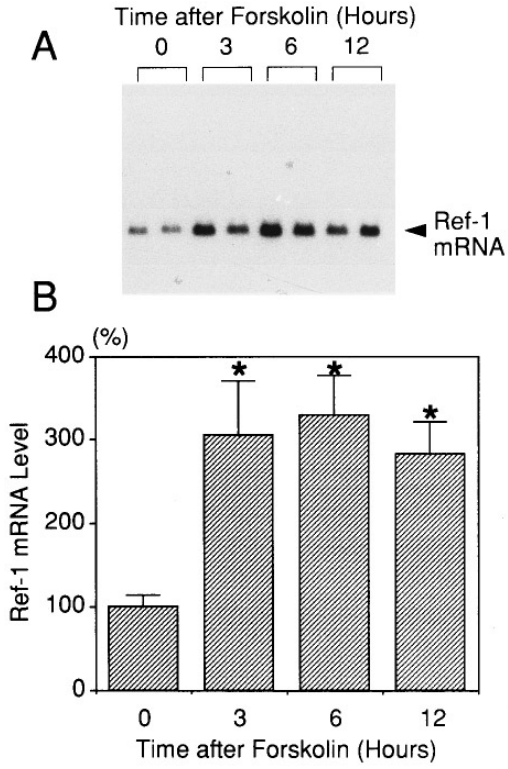

FIG. 2. Forskolin increases Ref-1 mRNA. FRTL-5 cells maintained in the basal medium for 4 days were exposed to 10 μM forskolin for 0, 3, 6, and 12 hours and then harvested for Northern blot analysis. The experiments were performed in quadruplicate. A representative autoradiograph is shown in A. The position of Ref-1 mRNA is indicated. Changes in the amounts of Ref-1 mRNA by forskolin are depicted in B. Values are expressed as percentage of the value before TSH treatment (time 0) and the means ± SE are indicated. * $p < 0.05$ (vs. time 0).

RESULTS

As shown in Fig. 1, Ref-1 mRNA was detected as a single band of approximately 1.6 kb. The size was compatible with the previous reports (11, 17) A substantial amount of Ref-1 mRNA was detected in FRTL-5 cells after culturing in the basal medium for 4 days. Addition of TSH to the medium significantly increased the level of Ref-1 mRNA 3 hours after the stimulation. This increase continued until 12 hours. The amounts of 28S and 18S ribosomal RNA blotted onto the membrane were almost similar in each lane, indicating equal application of RNA.

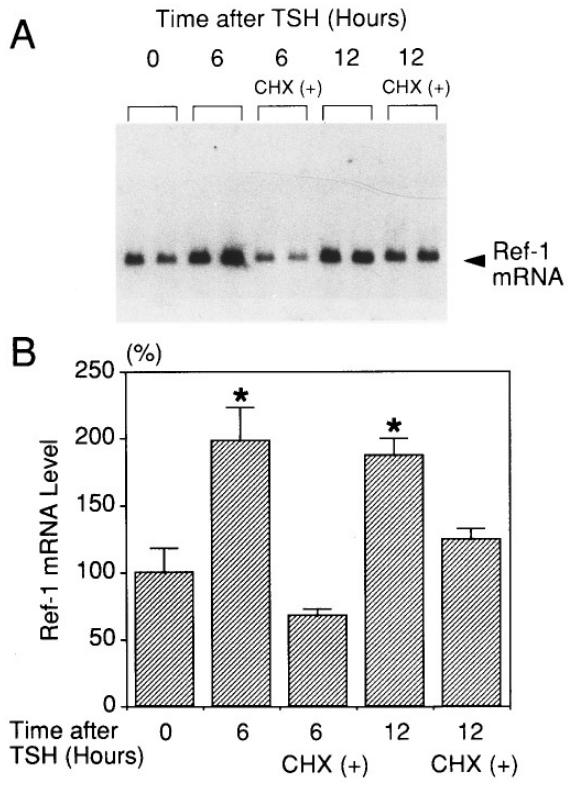

FIG. 3. CHX blunts the increase in Ref-1 mRNA by TSH. FRTL-5 cells maintained in the basal medium for 4 days were cultured in the presence or absence of 10 μg/ml CHX. After 15 min incubation, the cells were treated with 1 mU/ml TSH for 0, 6, and 12 hours and then harvested for Northern blot analysis. The experiments were performed in quadruplicate. A shows the representative autoradiograph. B shows changes in the amounts of Ref-1 mRNA. Values are expressed as percentage of the value before TSH treatment (time 0) and the means ± SE are indicated. * $p < 0.05$ (vs. time 0).

It is known that TSH action is mediated by several intracellular signalling pathways involving cAMP-protein kinase A and protein kinase C (18). To define the signalling pathway, we examined whether an adenyl cyclase activator, forskolin, could mimic the TSH action. As shown in Fig. 2, the increase in Ref-1 mRNA was also observed by treatment with forskolin, indicating that TSH increases Ref-1 mRNA via intracellular cAMP production.

We next examined whether the TSH effect on Ref-1 mRNA is mediated by a newly synthesized protein or not. As shown in Fig. 3, pre-treatment with protein synthesis inhibitor CHX blunted the increase in Ref-1 mRNA by TSH, indicating that *de novo* protein synthesis is required for the TSH action.

We then examined change in the amount of Ref-1 protein by TSH. As shown in Fig. 4, Western blot analysis showed a single band of approximately 35 kDa. This size was compatible with the previous reports (9, 17) The Ref-1 protein was also detected in the cells maintained in the basal medium. Treatment with TSH gradually increased the amount of Ref-1 protein until 12 hours in a time-dependent manner. These results indicate that the increase in Ref-1 mRNA by TSH is responsible for the increase in Ref-1.

DISCUSSION

The present study for the first time demonstrated that the expression of Ref-1 is increased by TSH in rat thyroid FRTL-5 cells. Increased expression of Ref-1 has been reported in two types of cells. In human HT29 colon cancer cells, exposure to hypoxia induces the expression of Ref-1 (12), and in HeLa cells, an increase in extracellular calcium concentration induces the expression (19). However, little is known concerning intracellular events leading to the activation of the gene. Our study shows that increase in intracellular cAMP production is involved in the activation of Ref-1 gene by TSH and that this activation is mediated by *de novo* synthesized protein.

We and others have shown that TSH rapidly and transiently increases the mRNA level of *fos* and *jun* family members (1-5). However, time course of the induction of the members at the protein level has not been reported. In mouse fibroblasts (Swiss 3T3), it has been demonstrated that various types of AP-1 proteins

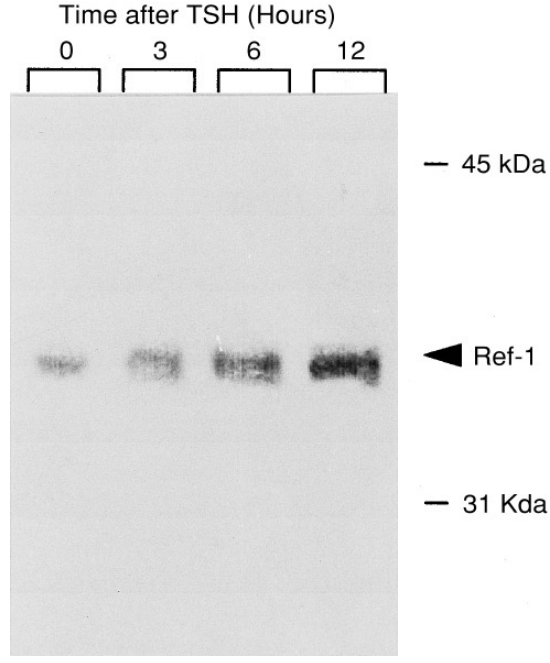

FIG. 4. TSH increases the Ref-1 protein in FRTL-5 cells. FRTL-5 cells maintained in basal medium for 4 days were treated with 1 mU/ml TSH for 0, 3, 6, and 12 hours. The whole cell extracts were prepared for Western blot analysis. The position of Ref-1 protein is indicated. The positions of carbonic anhydorase (31 kDa) and ovalbumin (45 kDa) are also indicated. Similar results were obtained from three separate experiments.

exist in the nucleus for 1 to 8 hours after the serum stimulation (20, 21). Since the patterns of increase in *fos* and *jun* family mRNAs in the mouse fibroblasts are similar to those in the thyroid cells, it is likely that AP-1 proteins are present in the thyroid cells for several hours after TSH stimulation. Our present result showed that Ref-1 protein increases within 3 hours after TSH, indicating that the induction of Ref-1 coincides with that of AP-1. Therefore, it is possible that increased Ref-1 is involved in the regulation of AP-1 in the thyroid cells. Ref-1 increases the DNA-binding activity of AP-1 by reducing the conserved cysteine in the molecule (11). Increased Ref-1 may play a role in gene regulation in the thyroid cells by regulating the activity of AP-1 which controls the expression of a variety of genes.

The function of Ref-1 may not be limited to the activation of AP-1, since Ref-1 has another function as a DNA repair enzyme (22). It is thus also called as APEX nuclease (9). This enzyme recognizes and repairs apurinic/apyrimidinic sites in genome DNA generated by spontaneous hydrolysis, radiation and oxidative stress etc (23). Our result showed the elevation of Ref-1 protein lasting 12 hours after TSH. This prolonged increase in Ref-1 protein may relate not only to regulation of AP-1 but also to its DNA repair function. Considering that TSH promotes DNA synthesis and proliferation of thyroid cells, and it also increases the production of hydrogen peroxide for the thyroid hormone synthesis (24), Ref-1 probably plays an important role in protection of the thyroid cells against DNA damage during cell growth and hormone synthesis. Thus, Ref-1 may play dual roles in gene regulation and DNA repair processes in the thyroid cells.

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