In Vivo Effect of Thyrotropin on Intracellular Translocation of Thyroid Peroxidase in Rat Thyroid Cells by an Indirect Immunofluorescence Method

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Abstract. The in vivo effect of thyrotropin (TSH) on the intracellular localization of thyroid peroxidase (TPO) in rat thyroid epithelial cells was examined by an indirect immunofluorescence method. The staining for TPO in the epithelial cells of normal rats appeared all over the cytoplasm, especially in the apical region. The injection of propylthiouracil for 3–10 days increased the staining in the apical region. The administration of L-thyroxine for 7–10 days to normal rats abolished the relatively high localization of TPO in the apical region, and resulted in TPO staining all over the cytoplasm. Six hours after TSH was injected into the thyroxine-treated rats, localization of TPO staining in the apical region was observed. These results suggest that TSH may play a role in the translocation of preexisting TPO to the apical region before TSH-induced biosynthesis becomes evident. (Endocrinol Japon 38: 89-95, 1991)

A NUMBER of biochemical and histochemical studies revealed that thyroid peroxidase (TPO) in normal human, rat and porcine thyroid glands is located in perinuclear cisternae, rough-surfaced endoplasmic reticulum and small apical vesicles. In addition to these intracellular organelles, some investigators demonstrated the presence of the enzyme on the external surface of microvilli [1–3], but the enzyme activity in such an area in normal rat [4], normal porcine [5] and normal human [6] thyroids seemed to be comparatively low. On the other hand, it is generally agreed that the peroxidase activity in microvilli and apical vesicles is stimulated by endogenous and exogenous TSH [2, 4, 7, 8] and that these enzymes participate in the stimulated thyroid hormone formation in hyperthyroids. Recently, it has been reported that the biosynthesis of TPO mRNA in FRTL cells [9] and TPO activity in primary cultured porcine thyroid cells [10, 11] are stimulated by incubation with TSH. Thus, increased synthesis of TPO and subsequent transport of the enzyme to the apical region are considered to be one of the TSH-dependent functions of thyroids. The TSH effect on the biosynthesis of TPO was shown to be mediated by cAMP [9, 11]. However, it is still unclear whether the TSH effect on the translocation occurs independently of the effect on the biosynthesis of TPO.

As reported previously [9, 11], the effect of TSH on the biosynthesis of TPO does not become evident within several hours after the TSH injection. Taking advantage of this fact, we examined whether the injection of TSH into T4-pretreated rats causes any change in the localization of TPO in the cells immediately after the injection. For this purpose, the indirect immunofluorescence method was used with anti-hTPO antibodies.
raised in rabbit serum.

**Materials and Methods**

**Materials**

T4 sodium salt and bovine TSH were purchased from Sigma Chemicals Co., and PTU was obtained from Tokyo Kasei Kogyo Co. FITC-conjugated goat anti-rabbit IgG antibody was purchased from Cappel Laboratories, U.S.A. An avidin-biotin peroxidase complex kit (Vectastain) was from Vector Laboratories. DEAE-cellulose (DE-52) and protein A-coupled Sepharose CL-4B were from Whatman Ltd. and Pharmacia Fine Chemicals, respectively. All other reagents were of analytical grade.

**Assay for peroxidase activity**

Peroxidase activity was determined with guaiacol as the second substrate as described previously [12]. One guaiacol unit (GU) of the enzyme was defined as the amount of enzyme that resulted in an increase in absorbance unit/s, under the conditons specified.

**Purification of hTPO**

Human TPO (hTPO) was purified from thyroids of patients with Graves' disease described elsewhere [13]. Briefly, TPO in microsomal fractions of human thyroids was solubilized with sodium cholate plus trypsin and purified with DEAE cellulose chromatography followed by HPLC (TSK-GEL, G 3000 SW, Toyo Soda, Japan). The RZ (A412/A280) was 0.25.

**Preparation of polyclonal antibodies to hTPO**

Female rabbits were injected subcutaneously at four to six sites on the back with 30 μg each of purified hTPO emulsified with a complete Freund's adjuvant (hTPO solution: adjuvant=4:6, v/v). The rabbits were boosted at 3 week intervals with 30, 50 and 50 μg each of emulsified with an equal volume of complete Freund's adjuvant. The production of anti-TPO antibodies was detected by passive haemagglutination, with microsome antigen test kits (Fuji Rebio Co., Tokyo). Serum was collected 2 weeks after the last boost and stored at −70°C. From the serum, an IgG-enriched fraction was prepared by ammonium sulfate (0–50%) fractionation, followed by ion exchange chromatography on DEAE-cellulose.

**Immunoprecipitation of TPO**

One hundred μl of variously diluted anti-hTPO rabbit IgG or normal rabbit IgG was mixed with 20 μl of hTPO solution containing 0.73 μgU and the mixture was incubated for 2 h at room temperature. To the mixture was added a mixture of 50 μl protein A-coupled Sepharose CL-4B and 50 μl PBS, and the mixture was further incubated for 2 h at room temperature. After centrifugation, an aliquot of the supernatant was used for the assay of peroxidase activity.

**Immunoblotting of TPO from rat thyroidal microsomes**

Microsomes prepared from rat thyroids by the method of Hosoya et al. [14] were treated with deoxycholate by the method of Yokoyama & Taurog [15], and subjected to SDS-PAGE in 10% separation gel under reduced conditions, followed by transfer to nitrocellulose membrane and immunostaining as described previously [16]. Briefly, the membrane blotted was incubated for 1 h at room temperature with 500-fold diluted anti-hTPO antiserum and then stained with the peroxidase-conjugated avidin-biotin-complex and 4-chloro-1-naphthol as substrate.

**Treatment of animals**

Male rats of Wistar strain (250–300 g) were divided into four experimental groups. One group consisted of at least three rats. Group 1 (control rats): the animals were injected ip with 0.5 ml of the vehicle (0.9% NaCl containing 30% ethanol) daily for 10 days. Group 2: The animals were injected daily with PTU (1.5 mg, ip) for 10 days. Group 3: The animals were injected daily with T4 (5 μg, s.c.) for 1–20 days. Each rat in groups 1 to 3 was sacrificed 1 day after the last injection. Group 4: The animals pre-treated with T4 (5 μg, s.c., 10 days) were given TSH (1 IU, s.c.) at various times before killing.

**Preparation of tissue sections**

The thyroid gland, together with the trachea, was removed under ether anesthesia, quickly

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**Abbreviations used are:** hTPO, human thyroid peroxidase; TSH, thyrotropin; T4, L-thyroxine; PBS, 0.01 M sodium phosphate buffer (pH 7.4) containing 0.9% NaCl; PTU, 2-propyl-6-thiouracil; T3, 3,3',5-triiodothyronine; rTPO, rat thyroid peroxidase; TPO, thyroid peroxidase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
frozen with embedding matrix (Tissue TeK, O.C.T. Compound, Miles laboratories, U.S.A.) in dry ice-acetone, and stored at -70°C until use. The frozen samples were cut into 5 µm sections, air-dried on glass slides, and fixed in periodate-lysine paraformaldehyde fixative [17] for 5 min at 4°C. After fixation, the slices were rinsed in three changes of PBS and used for indirect immunofluorescence study as described below or stained with hematoxylin and eosin for morphological observations.

Indirect immunofluorescence
The fixed sections were incubated with 10% nonimmunized normal goat serum for 1 h at room temperature in a moist chamber to decrease nonspecific binding, and then incubated with anti-TPO antisera diluted (1:40) with PBS containing 1% bovine serum albumin at 4°C overnight. The samples were rinsed in five changes of PBS containing 0.02% Tween 20 and incubated with FITC-conjugated goat anti-rabbit IgG antibodies diluted (1:40) with PBS containing 1% bovine serum albumin in the dark. The slides were then rinsed in five changes of PBS containing 0.02% Tween 20 and one change of PBS, mounted in glycerol, coverslipped and examined with a fluorescence microscope (Nikon Optiphoto System, Nikon Co., Yokohama, Japan). In the control experiments, normal rabbit serum was used instead of anti-TPO antiserum.

Results
Cross-reactivity of anti-hTPO antibodies with rTPO
Various diluted rabbit anti-hTPO antiserum was examined to precipitate purified hTPO (Fig. 1A-a) and rTPO solubilized from rat thyroid microsomes (Fig. 1A-b), indicating that the antiserum cross-reacts with rTPO. As shown in Fig. 1B, immunoblotting of rat and human thyroid microsomal TPO with anti-hTPO antibodies indicates the presence of 100 kDa protein bands which corresponds to those of porcine TPO reported previously [15]. These results indicate that rabbit anti-hTPO antibody cross-reacts with rTPO.

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Binding ability of anti-hTPO polyclonal antibodies to rTPO and hTPO by immunoprecipitation method and immunoblotting method. (A) Partially purified TPO of human or rat thyroid microsomes was immunoprecipitated with anti-hTPO (•) or control (○) IgG with various dilutions, and TPO activities of the supernatant were measured as described in Materials and Methods. (a) hTPO, (b) rTPO. (B) Immunoblotting of TPO in microsomes of rat and human thyroids. Proteins in thyroid microsomes were blotted to nitrocellulose membrane and then immunostained with anti-hTPO antibodies and peroxidase-conjugated avidin-biotin complex.
Fig. 2. Immunofluorescence localization of TPO in thyroids of control (normal) and PTU-, T₁, and TSH-treated rats. 
(a) Normal rats; (b) rats treated with PTU (1.5 mg/day) for 10 days. (c) Rats treated with T₁ (5 µg/day) for 10 days. 
(d) Rats treated with T₁ (5 µg/day) for 10 days. TSH (1 IU/rat) was injected into rats previously treated 
with T₁ (5 µg/day) for 10 days, and rats were sacrificed 2 h (d), 6 h (e) and 24 h (f) after the injection of TSH. 
800×
Indirect immunofluorescence staining of TPO in thyroid glands of control (normal), PTU-, T4- and TSH-treated rat

Figure 2a shows the staining of TPO in thyroids of control (normal) rats. Immunofluorescence appeared all over the cytoplasm, especially in the apical region.

PTU is an inhibitor of TPO and the administration of PTU blocks the formation of thyroid hormones, leading to stimulated secretion of TSH from the pituitary gland and subsequent hyperplasia of the thyroid gland. These changes became apparent within 3–5 days after the PTU injection and 'papillary growth' of the follicular epithelium cells was observed in most cells after 7–10 days (Fig. 2b). TPO staining became especially evident in the apical area.

Administration of T4 causes repression of TSH secretion from the pituitary gland. The follicular lumens were gradually enlarged, with decreasing cell height. As shown in Fig. 2c, fluorescence for TPO became evident in the perinuclear region, but staining of TPO in apical areas was not so remarkable.

When rats pretreated with T4 for 10 days were injected with TSH, the distribution of TPO in the cytoplasm of thyroid cells was changed quickly. Within 2 h after the injection, localization of TPO staining became evident in the apical area rather than in the basal area (Fig. 2d). Most of the intracellular TPO seemed to move to the apical area within 6 h after the injection of TSH (Fig. 2e). After 24 h, the height of follicular epithelial cells and the TPO stainability over the cytoplasm were markedly increased, becoming similar to those of normal thyroids (Fig. 2f).

These results suggest that de novo synthesis of TPO and its translocation to the apical region took place at this time.

Discussion

In the present study, TPO in thyroid cells was stained by an indirect immunofluorescence method, taking advantage of anti-hTPO antibodies which cross-react with rTPO. Observed intracellular TPO distribution was in general in agreement with previous histochemical results [4]. As compared with the electron microscopic observation, this method, employing frozen sections of thyroid tissues, requires only a shorter time to manoeuvre. In addition, the loss in antigenicity due to fixation with paraformaldehyde occurs in a lesser degree compared with the loss in peroxidase activity. Thus, this method is especially useful for clinical purposes. In fact, some applications of this method for diseased thyroid tissues are now under way.

The characteristic feature found in the present study by the technique used is that the administration of TSH to T4-pretreated rats caused a distinct change in the localization of TPO: TPO evenly distributed in the cytoplasm after T4 treatment moved to the apical area after the TSH administration (Figs. 2c-2f). The translocation was found to occur as early as 6 h after TSH injection. According to previous papers, the TSH-induced increase in TPO mRNA [18], TPO protein [8] and TPO activity [10, 11] showed a lag time of more than 8–10 h in cultured pig thyroid cells. Thus, the TSH effect mentioned above seems to indicate the stimulation of translocation of preexisting TPO in the cytoplasm soon after TSH injection, although other possibilities, such as a change in the immunohistochemical stainability of the enzyme, cannot be completely excluded.

There is ample evidence indicating that the effect of TSH on the biosynthesis is mediated by cAMP [9, 11, 19], but it is still unclear what mediates the effect on the translocation of TPO. Recently TSH was reported to act on the phosphatidyl inositol system [20–23]. It is well established that the system is related to Ca2+ mobilization in many cells [24, 25] and in fact the increase in the intracellular Ca2+ concentration by TSH was reported by Sheela Rani et al. [26] and Corda et al. [27]. This raises the possibility that the effect of the translocation of TPO mentioned above is mediated by Ca2+, since Ca2+ is known to play an important role in the intracellular transportation and secretion of proteins in various kinds of cells.

It was previously reported that hypophysectomy of rats deprived their thyroids of TPO activity within 3 weeks [2]. The elimination of TSH from the culture medium of FRTL-5 resulted in the disappearance of TPO antigens from both the surface and cytoplasm of the cells within 72 h [8]. These results exhibit a striking contrast to the present experimental results which show that TPO stainability was not diminished by suppression of TSH secretion after T4 administration even for 20 days, although its location in the cytoplasm was
affected. This suggests that unknown factors other than TSH may be related to the biosynthesis and degradation of TPO. In this connection, it is of interest to note that the expression of the Tg gene was recently reported to be stimulated by estrogen [28] and hydrocortisone [29].

In conclusion, it was found by the immunofluorescence method that quick translocation of preexisting TPO in cytoplasm toward the apical region is caused by enhanced secretion of TSH, independent of the biosynthesis of TPO.

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References


