Characterization of Thyroid Follicular Cell Apical Plasma Membrane Peroxidase Using Monoclonal Antibody

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Abstract

Thyroid peroxidase (TPO) located in the apical plasma membrane of follicular cells was investigated by means of a membrane-immunofluorescent technique. The epitope of TPO recognized by a murine monoclonal antibody (mAb 30.1.2) was identified on the apical membrane surface. Trypsinization removed TPO immunoreactivity and enzymatic activity after 60 min of incubation at 37°C. The epitope reappeared on the apical membrane surface after short term culture for 120 min without the addition of TSH. With TSH the time required for reappearance was only 30 min. TPO activity was regenerated under both conditions. Since dibutyryl cyclic AMP could not accelerate the reappearance of the epitope, it was thought that TPO reappearance is mediated by other than the adenylate cyclase-cyclic AMP system.

Thyroid peroxidase (TPO) is believed to play an essential role in the biosynthesis of thyroid hormone because of its ability to catalyze the iodination of tyrosine residues in thyroglobulin (Tg). Cytochemical studies using diaminobenzidine (DAB) staining for TPO have shown that TPO is distributed in exocytotic vesicles as well as the apical plasma membrane (Strum and Karnovsky, 1970; Tice and Wollman, 1972; Ofverholm and Ericson, 1984), which is regarded as the principal site of iodination (Wollman and Wodinsky, 1955; Stein and Gross, 1964; Ekholm and Wollman, 1975). However, some workers have disagreed with the localization of this enzyme in the apical plasma membrane both cytochemically and biochemically (Hosoya et al., 1971, 1972; De Wolf et al., 1978).

A murine monoclonal antibody (mAb) to porcine TPO (mAb 30.1.2) has recently been produced and applied for the immunohistochemical analysis of TPO localization. Although a small amount of TPO could be occasionally detected in the apical plasma membrane including microvilli, it has remained to be studied whether TPO located in the apical plasma membrane increases by stimulating with TSH (Nakagawa et al., 1985a, b). Therefore, we attempted to demonstrate the localization of TPO in the apical plasma membrane by a direct method...
such as a membrane-immunofluorescent technique using partially disrupted follicles ("half-melon"-like structures) (Khoury et al., 1984) instead of cytochemical techniques. Successful identification and characterization of TPO located in the apical plasma membrane are reported here.

**Materials and Methods**

**Preparation of thyroid follicles**

Fresh porcine thyroid glands were obtained from a local abattoir and transported to the laboratory in ice-cold Dulbecco's salt solution without CaCl₂ and MgCl₂ (pH 7.4) [PBS (--) ; Nissui Seiyaku Ltd., Tokyo]. They were freed from extraneous tissues, chopped into small pieces, washed three times with PBS (--) and incubated for 60 to 120 min at 37°C with 2 mg/ml collagenase (Wako Pure Chemicals Ltd., Tokyo) in Medium 199 (GIBCO Laboratories, Grand Island, NY) containing 5% fetal calf serum (FCS; GIBCO Laboratories) (Nitsch and Wollman, 1980). After vigorous pipetting and 1g sedimentation for 5 min, supernatants rich in partially disrupted follicles were collected, and then erythrocytes were lysed in 0.83% ammonium chloride buffer. For some experiments, the follicles were further digested with 0.25% trypsin (GIBCO Laboratories) for 30 to 60 min. FCS was added up to 10% (vol/vol) to stop trypsin digestion, and then the follicles were washed three times with Medium 199 containing 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) (1% BSA-199). More than 90% of cells composed of the disrupted follicles were viable when they were judged by trypan blue exclusion test.

**Short term culture**

To study the appearance of TPO immunoreactivity on the apical membrane surface, trypsinized follicles in 2 ml of Minimal Essential Medium (MEM; GIBCO Laboratories) containing 1% BSA (1% BSA-MEM) were cultured in a 5% humidified incubator at 37°C for 15 to 120 min. Bovine TSH and dibutyryl cyclic AMP (Sigma Chemical Co.) were added to the culture media at a final concentrations of 0.1 U/ml and 1 mM, respectively. After incubation, the follicles were subjected to membrane-immunofluorescent analysis.

**Antibodies**

mAb 30.1.2 raised in BALB/c mouse and specific for porcine TPO has been reported elsewhere (Nakagawa et al., 1985b). Our previous immunohistochemical study suggested that the endoplasmic reticulum and perinuclear cisternae bear an epitope recognized by mAb 33.1.1 (our unpublished data), therefore we used mAb 33.1.1 raised in BALB/c mouse as a control. Two other mAbs (15.1.1 and 16.3.2) to human Tg produced in C3H/He mouse were previously reported (Kotani et al., 1985). These mAbs cross-reacted with porcine Tg (our unpublished data). All four mAbs have the same isotype of IgG1 and a κ-light chain.

Fluorescein isothiocyanate (FITC)-labeled goat anti-murine IgG antibody (purified with an affinity column) as a secondary antibody was purchased from TAGO Inc. (Burlingame, CA).

**Indirect immunofluorescence**

Indirect immunofluorescence with follicular cells was previously described (Kotani et al., 1982). Briefly, approximately 5×10⁶ of partially disrupted follicles suspended in 200 µl of mAb (50 µg/ml in Medium 199) were incubated on ice for 30 min with two vigorous agitations. After washing three times, these were further incubated on ice for 30 min in 200 µl of FITC-labeled goat anti-murine IgG antibody (at 1:5 dilution). Final cell pellets were washed three times with Medium 199, and then 70 to 100 of partially disrupted follicles were evaluated for their specific fluorescence under a fluorescent microscope (Model BHF, Olympus Ltd., Tokyo). Phase-contrast showed a three-dimensional structure (a "half-melon"-like structure) (Khoury et al., 1984).

**DAB staining for TPO activity on apical membrane surface**

To demonstrate TPO activity on the apical membrane surface, disrupted follicles were studied. Follicles were kept in cold Tris-HCl-Saline containing 50 mM Tris-HCl (pH 7.6) and 0.15 M NaCl on ice before mixing with an equal volume of substrate solution containing 50 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 0.15% DAB (Wako Pure Chemicals Ltd., Tokyo) and 0.006% H₂O₂ (Strum & Karnovsky, 1970; Matsukawa et al., 1981). DAB oxidation products on the apical membrane surface were examined under a
light microscope at room temperature. During the initial 20 min, DAB oxidation products were seen only on the apical membrane surface, but subsequently the perinuclear region slowly began to show brown oxidation products. Therefore, unless otherwise specified, it was usually judged 10 min after mixing follicles with substrate solution whether DAB oxidation products were present or not. The amount of DAB oxidation product on the apical membrane surface was judged from the degree of color development. Very brown, brown and fairly brown colors were regarded as indicating large, small and very small amounts of DAB oxidation product, respectively.

Results

In order to determine whether TPO is located in the apical plasma membrane of follicular cells, membrane-immunofluorescent studies were done using partially disrupted follicles prepared by treatment with collagenase. Specific fluorescence on the apical membrane surface was observed to be as shown in Fig. 1A. Ten to 20% of disrupted follicles showed specific fluorescence on their apical membrane surface. Three mAbs (33.1.1, 15.1.1 and 16.3.2) as the control demonstrated no fluorescence on the follicular cell surface (Table 1). These results suggested that TPO was located in the apical plasma membrane and that its epitope recognized by mAb 30.1.2 was exposed to the follicular lumen.

To characterize TPO located in the apical plasma membrane, follicles obtained by collagenase treatment were further digested with trypsin and then used to examine the immunoreactivity of TPO exposed to the follicular lumen. Digestion for 30 min with trypsin was found to be insufficient to remove the epitope of TPO on the apical membrane surface, but an additional 30 min of digestion could remove the epitope completely (data not shown).

To determine whether the epitope of TPO reappears on the apical membrane surface, partially disrupted follicles trypsinized for 60 min were incubated at 37°C, then the epitope on the apical membrane surface was examined for 120 min. Although no specific fluorescence on the apical membrane surface was observed until after 60 min of incubation, 5 to 10% of disrupted follicles showed specific fluorescence at 120 min (Table 2, upper row). Further investigation

Table 1. Results of indirect immunofluorescent study

<table>
<thead>
<tr>
<th>mAb isotype</th>
<th>Specificity</th>
<th>Fluorescence</th>
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<tr>
<td>30.1.2 IgG1</td>
<td>BALB/c anti-porcine TPO</td>
<td>+</td>
</tr>
<tr>
<td>33.1.1 IgG1</td>
<td>BALB/c anti-porcine microsomal antigen excluding TPO</td>
<td>-</td>
</tr>
<tr>
<td>15.1.1 IgG1</td>
<td>C3H/He anti-human Tg</td>
<td>-</td>
</tr>
<tr>
<td>16.3.2 IgG1</td>
<td>C3H/He anti-human Tg</td>
<td>-</td>
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Specific fluorescence on the apical membrane surface of partially disrupted follicles was examined after incubation with mAb and with FITC-labeled goat anti-murine IgG antibody. +, specific fluorescence observed; –, not observed.
Table 2. Enhancement of TPO reappearance after treating with TSH

<table>
<thead>
<tr>
<th>TSH</th>
<th>Incubation time (min)*</th>
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<tbody>
<tr>
<td></td>
<td>15</td>
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<tr>
<td>−</td>
<td>NT**</td>
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<tr>
<td>+</td>
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* Partially disrupted follicles digested with trypsin for 60 min were incubated in 1% BSA-MEM at 37°C with or without the addition of 0.1 U/ml bovine TSH.
** Not tested.
# Percentage of disrupted follicles showing specific fluorescence. The range resulting from three experiments is shown.

was then undertaken to determine whether TSH is able to accelerate the reappearance of the epitope on the apical membrane surface. When 0.1 U/ml TSH was added to the culture media, the epitope of TPO reappeared as early as 30 min after the initiation of incubation on a low percentage of disrupted follicles and the percentage gradually increased with time (Table 2, lower row). When dibutyryl cyclic AMP was added to the culture media at a concentration of 1 mM, maximally 20 mM, to study its influence on the reappearance of the epitope on the apical membrane surface, no effect was observed (data not shown). These experiments were repeated three times and all of them gave essentially the same result.

Finally, in order to estimate TPO activity on the apical membrane surface, it was examined whether DAB could be oxidized on the apical membrane surface. Disrupted follicles obtained by collagenase treatment showed a small amount of DAB oxidation products on the apical membrane surface but no DAB oxidation products were observed after 60 min digestion with trypsin (data not shown). As shown in Table 3, when trypsin-treated follicles were stimulated with TSH and incubated with both DAB and H₂O₂, a small amount of DAB oxidation products on the apical membrane surface was observed 5 min after the initiation of the reaction and increased with time (Fig. 2A). Follicles incubated with only DAB showed faint DAB oxidation products on the apical membrane surface at 15 min but those incubated with only H₂O₂ did not. DAB oxidation was completely inhibited by 0.1% NaN₃ (Fig. 2B). Disrupted follicles cultured without the addition of TSH for 60 min began to oxidize DAB 15 min after initiation. TPO in the apical plasma membrane was thought to retain its inherent catalytic capability as peroxidase.

Discussion

Our present immunofluorescent study using mAb 30.1.2 and partially disrupted thyroid follicles showed direct evidence that TPO is located in the apical plasma membrane and revealed that partially disrupted

Table 3. Oxidation activity of TPO located in apical plasma membrane

<table>
<thead>
<tr>
<th>Substrate mixture</th>
<th>Reaction time (min)</th>
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<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>(Cultured with TSH)</td>
<td></td>
</tr>
<tr>
<td>DAB+H₂O₂</td>
<td>+</td>
</tr>
<tr>
<td>DAB</td>
<td>−</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>−</td>
</tr>
<tr>
<td>DAB+H₂O₂+0.1% NaN₃</td>
<td>−</td>
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</tbody>
</table>

(Cultured without TSH)

<table>
<thead>
<tr>
<th>Substrate mixture</th>
<th>Reaction time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>DAB+H₂O₂</td>
<td>−</td>
</tr>
</tbody>
</table>

Reaction mixture containing partially disrupted follicles, DAB and H₂O₂ was examined for DAB oxidation products on the apical membrane surface of follicular cells under a light microscope at room temperature. Disrupted follicles were treated with trypsin for 60 min and cultured with or without TSH for 60 min. All combinations were tested three times separately and nearly the same results were obtained. ++, a large amount of DAB oxidation products; +, a small amount; ±, a very small amount; −, no product.
Fig. 2. DAB oxidation products on the apical membrane surface of a disrupted follicle obtained after stimulation with TSH. A: A partially disrupted follicle 15 min after the initiation of reaction shows diffuse DAB oxidation products on its apical membrane surface (arrow). B: DAB oxidation is completely inhibited by 0.1% NaN₃. ×2,100.

The epitope recognized by mAb 30.1.2 on the apical membrane surface was removable from the cell surface by trypsinization and its reappearance took place after 120 min of short term culture. The reappearance of the epitope is either ascribable to newly synthesized TPO or to the movement of preexisting TPO to the outer surface of the plasma membrane. Although the latter possibility seems more likely because the turnover time of the epitope was only 30 min with TSH stimulation, further study is necessary to clarify this aspect.

While dibutyryl cyclic AMP as well as TSH has been shown to induce TPO activity in vitro (Magnusson and Rapoport, 1985), the reappearance of the epitope on the apical membrane surface was not accelerated by the addition of dibutyryl cyclic AMP. This result indicates that the TSH action on the reappearance of the epitope was not mediated by the adenylate cyclase-cyclic AMP system (Dumont et al., 1981). DAB oxidation products were observed on the apical membrane surface of partially disrupted follicles 5 min after the initiation of the reaction, when both DAB and H₂O₂ were added. In the absence of H₂O₂, a very small amount of DAB oxidation products was deposited at 15 min. The latter observation may support the view that H₂O₂ is generated on the apical membrane surface through an enzyme system located in the apical plasma membrane (Bjorkman and Ekholm, 1984). The addition of NaN₃ to the reaction mixture inhibited DAB oxidation completely, in keeping with our previous report (Ohtaki et al., 1985). Disrupted follicles cultured without the addition of TSH showed a small amount of DAB oxidation products on their apical membrane surface 15 min after the initiation of the reaction. This observation suggests either that 60 min incubation without TSH is enough for a small amount of TPO activity to regenerate or that an additional 15 min incubation at room temperature makes TPO activity reappear. In any case, TPO activity
on the apical membrane surface of disrupted follicles treated with trypsin was regenerated by culturing with TSH. This activity corresponds closely to that of the epitope recognized by mAb 30.1.2.

In our previous immunohistochemical study, only a small amount of TPO could be detected in the apical plasma membrane (Nakagawa et al., 1985a). Our present membrane-immunofluorescent analysis showed specific fluorescence on the apical membrane surface of 10 to 20% of the disrupted follicles prepared by treating with collagenase. And the number of fluorescence-positive partially disrupted follicles increased as much as 30 to 40% after stimulation with TSH for 120 min. These results suggest that there is a little TPO in apical plasma membrane in the physiological state and it increases with additional stimulation of TSH.

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References


Ohtaki, S., H. Nakagawa, S. Nakamura, M.


