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Biosynthesis, post-translational modifications, maturation, and physiological function of human thyroid peroxidase**

Biosynteza, modyfikacje postranslacyjne, dojrzewanie i fizjologiczna funkcja ludzkiej peroksydazy tarczycowej

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Summary

Apart from being the major target autoantigen in autoimmune thyroid disease, thyroid peroxidase (TPO) is the key enzyme, together with DUOX proteins, responsible for the thyroid hormone generation. Newly synthesized TPO is processed in the endoplasmic reticulum, undergoing N-glycosylation, heme fixation, and transient interaction with molecular chaperones. Then, while transporting through the Golgi apparatus, O-glycans are attached to the TPO molecule, whereas N-linked oligosaccharides undergo further modifications. After exiting the Golgi, but before it reaches the cell surface, the propeptide is removed. TPO forms dimers, however, the mechanism of this process is still unknown. Only part of the synthesized TPO reaches the cell membrane, whereas partially or totally misfolded forms of TPO are degraded in the endoplasmic reticulum by the proteasome or a plasminogen-like protease, respectively.

Key words: thyroid peroxidase, thyroid hormones, glycosylation, propeptide, heme prosthetic group

Streszczenie

Peroksydaza tarczycowa (TPO) jest jednocześnie głównym autoantygenem w autoimmunologicznych chorobach tarczycy i kluczowym enzymem, wraz z białkami DUOX, odpowiedzialnym za syntezę hormonów tarczycy. Jeszcze na poziomie retikulum endoplazmatycznego nowo zsyntetyzowana TPO przechodzi proces N-glikozylacji i włączania hemu oraz przejściowo oddziałuje z białkami opiekuńczymi (chaperonami). Następnie podczas transportu przez aparat Golgiego do cząsteczki TPO dołączane są O-glikany, natomiast oligosacharydy połączone z białkiem wiązaniem N-glikozydowym poddawane są dalszym modyfikacjom. Po opuszczeniu aparatu Golgiego, ale przed dotarciem na powierzchnię komórki, z peroksydazy zostaje usunięty propeptyd. TPO tworzy dimery, jednak mechanizm tego procesu jest nadal nieznany. Tylko część zsyntetyzowanego białka dociera do błony komórkowej, natomiast cząsteczki z częściowym lub całkowitym zaburzeniem struktury przestrzennej są degradowane w retikulum endoplazmatycznym przez odpowiednio proteasom lub proteazę podobną do plazminogenu.

Słowa kluczowe: peroksydaza tarczycowa, hormony tarczycy, glikozylacja, propeptyd, hemowa grupa prostetyczna

INTRODUCTION

Since 1985 we have known that human thyroid peroxidase (TPO), previously known as the thyroid microsomal antigen, is a target for the autoimmune response in autoimmune thyroid diseases (1). The majority of patients' autoantibodies to thyroid peroxidase are directed to two discontinuous determinants on the protein surface. Several approaches have been taken by our and other laboratories to localize the autoantibody binding regions on TPO. These studies provided a greater insight into the nature of the autoimmune response to the thyroid. In a few recent reviews the current knowledge on TPO as an autoantigen was summarized (2-5). Here, we focus on the biochemical aspects of thyroid peroxidase, especially on its maturation process, trafficking and participation in thyroid hormone biosynthesis.

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PROTEIN STRUCTURE AND EXPRESSION

The human TPO gene, that was cloned in 1987 (6-8), is located on chromosome 2 (region pter-p12) and it is composed of 17 exons and 16 introns (9). The TPO expression is controlled by thyroid-specific factors, namely TTF-1, TTF-2, and Pax-8 (reviewed in Ref. 10-12). Till now several isoforms of TPO cDNA were identified, cloned and characterized (reviewed in Ref. 2, 10).

TPO belongs to the animal peroxidase protein family together with myeloperoxidase (MPO), lactoperoxidase (LPO), eosinophil peroxidase (EPO) (reviewed in Ref. 13, 14). TPO, a 933 amino-acid residues polypeptide, contains ectodomain, single membrane-spanning region and short intracellular tail (15). The dominant part of the protein, called ectodomain, consists of three distinct domains, namely MPO-like domain with high homology to myeloperoxidase (residues 142-738), CCP-like domain similar to complement control protein (residues 739-795), and EGF-like domain that is homologous to the epidermal growth factor (residues 796-841) (15). A three-dimensional computer model of TPO ectodomain was constructed in our laboratory, however, the localization of all TPO domains relatively to each other is still unknown (15). Till now the attempts to solve the TPO structure by X-ray crystallography ended in failure (16, 17). On the contrary, the crystal structure of other two members of the animal peroxidase family, namely MPO (18, 19) and LPO (20), has been already determined.

POST-TRANSLATIONAL MODIFICATIONS

The TPO molecule undergoes a few modifications while biosynthesis, that are glycosylation, heme incorporation, propeptide removal, and dimer formation (fig. 1). TPO ectodomain contains five predicted N-glycosylation sites in the amino-acid sequence (Asn129, Asn307, Asn342, Asn478, and Asn569) (6-8). However, glycosylation at position 478 probably does not occur because the X residue of the Asn-X-Thr consensus sequence is proline (21). It is well documented that N-linked oligosaccharides are added to the nascent protein in the endoplasmic reticulum (ER) (reviewed in Ref. 22). At this stage, the glycoprotein contains high mannose oligosaccharides that are sensitive to endoglycosidase H (Endo H) digestion. During the passage through the Golgi apparatus, glycoprotein undergoes a final process of N-glycosylation resulting in the transformation of Endo H-sensitive high mannose oligosaccharides to Endo H–resistant complex oligosaccharides. Thus, susceptibility to Endo H digestion is used to distinguish between high-mannose and complex oligosaccharides and help in differentiation whether the protein is before or after transit through the Golgi apparatus (reviewed in Ref. 22).

N-glycans content in human TPO was debated. The hypothesis, that TPO acquires complex oligosaccharides during maturation, was confirmed in research on TPO purified from human thyroid (23, 24), full-length TPO produced in insect (25) and CHO cells (26, 27), TPO ectodomain expressed in insect (24, 28) and CHO cells (28). Deglycosylation analysis of intracellular and

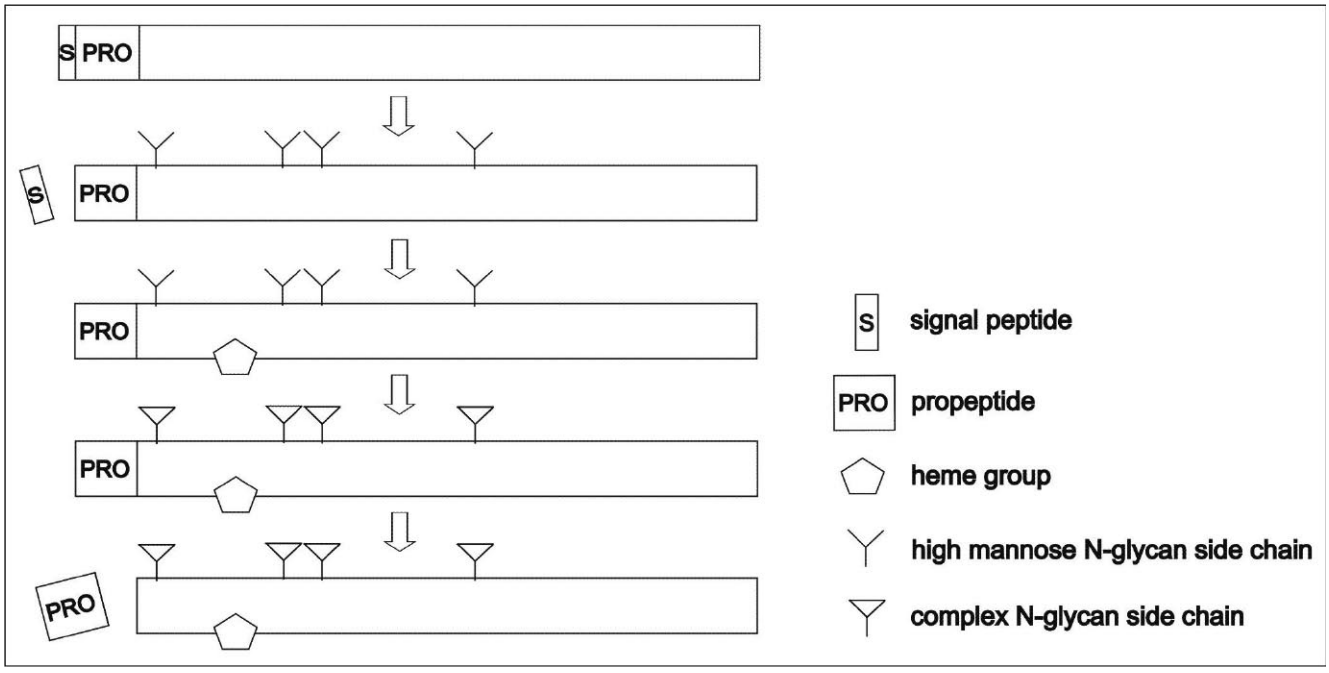

Fig. 1. TPO processing scheme. High mannose oligosaccharide side chains and heme group are incorporated to the TPO translation product in the endoplasmic reticulum. Signal peptide cleavage occurs also in this compartment (like in MPO) (reviewed in Ref. 47) or later during TPO transport to the cell surface (no data available). Some O-glycan side chains (not shown in this scheme), as well as complex N-glycan chains, are added to the TPO polypeptide in the Golgi apparatus. The propeptide is removed after its transit through the Golgi apparatus but before it reaches the plasma membrane. Mature TPO forms dimers (not shown in this scheme), however, the localization of dimerisation process in the cell is still unknown.

cell surface fractions of TPO expressed in CHO cells showed that, inside the cell, this protein contains only high mannose-type structures, whereas TPO at plasma membrane bears complex-type structures (26). However, there are some reports suggesting that TPO contains only high mannose structures regardless of whether the protein is expressed in CHO cells (29) or is purified from thyroid gland (30). Additionally, other studies showed that TPO exiting ER does not necessarily acquire endoglycosidase H resistance (31). Human TPO produced in MDCK (Madin-Darby Canine Kidney) cells was Endo H-sensitive inside the cell and at the cell membrane. The same protein transfected to PC Cl3 (rat thyrocytes) was predominantly sensitive (about 75%) to Endo H digestion (being integrated to the plasma membrane) indicating the lack of conversion to complex carbohydrates upon passage through the Golgi complex (31). Nevertheless, in the same study it was observed that TPO ectodomain secreted to the medium by CHO cells was resistant to the endoglycosidase H digestion. The phenomenon of not acquiring Endo H resistance upon trafficking through the Golgi was earlier observed and it may be explained by the inaccessibility of high mannose oligosaccharides in the tertiary and quaternary structure (32). However, it needs explanation why in some studies the modification of these inaccessible high mannose N-glycans is possible.

N-linked oligosaccharide moieties serve various functions, e.g., stabilize the proteins against denaturation and proteolysis, improve solubility, modulate immune response, regulate protein turnover, stabilize the structure of proteins, and promote their adequate sorting and quality control (reviewed in Ref. 22, 33). The role of these units in TPO was examined. After Endo H treatment, the specific activity of TPO purified from human thyroid, measured by guaiacol and iodide oxidation assays, was reduced about 4 times in comparison to non-deglycosylated protein (30). The authors confirmed that the aggregated state of the molecule, that could be induced by the glycans removal, was not responsible for this decrease (30). They assigned this reduction to the modification of the molecule's conformation at the enzymatic site (30). Kiso et al. (23) also demonstrated that enzymatic activity of native TPO treated with endoglycosidase H was significantly lower, when measured with guaiacol assay, but no effect was observed in the iodide oxidation assay. In further studies, the inhibition of N-glycosylation using tunicamycin (Tu) reduced by half enzymatic activity of human TPO at the cell surface in CHO cells (26). When the cells were treated with deoxymannojirimycin (dMM) which leads only to high mannose-type structures formation, the activity was not (or only slightly) reduced (26). The cell surface expression of newly synthesized TPO, after treatment with Tu, was almost totally blocked (by 95%) what may explain the decrease in enzymatic activity. All in all, it seems that N-glycans play an essential role in the intracellular trafficking and enzymatic activity of TPO. Additionally, the reactivity of unglycosylated TPO with anti-TPO antibodies, namely human autoimmune antibodies (23, 29, 30), mouse monoclonal antibodies (26, 30), and rabbit polyclonal antibodies (30), was determined. The conclusion was that the tertiary structure of epitopes recognized by anti-TPO antibodies is not markedly changed (23, 29, 30), however, the opposite observations were made by Fayadat et al. (26). Finally, as will be discussed later, N-linked oligosaccharides mediate the interaction between TPO and molecular chaperones (calnexin, calreticulin, and BiP) (34, 35).

The incorporation of O-glycans to human TPO was also analyzed. TPO purified from human thyroid (23), full-length human TPO expressed both in insect (25) and CHO cells (26) undergoes O-glycosylation. Opposite conclusions were drawn from the experiments conducted on full-length human TPO (29) and human TPO ectodomain (28), both expressed in CHO cells. In more detailed study, after separation of intracellular and cell-surface TPO, it was shown that only TPO localized in the latter fraction bears O-glycans (26). This finding was in agreement with the fact that O-glycosylation process occurs in the Golgi apparatus (reviewed in Ref. 33). O-glycans removal from native TPO, isolated from human thyroid gland, probably has no impact on enzymatic activity and interaction with autoantibodies (23). The treatment of the TPO-expressing CHO cells with phenyl-α-GalNAc, a specific inhibitor of O-glycosylation, only slightly altered TPO traffic to the cell surface and activity of this protein, nevertheless, the role of O-linked carbohydrates in the protection of TPO against proteolysis cannot be excluded (26).

The nature of the heme prosthetic group of TPO is still unclear. It was postulated that hog TPO contains other heme group than ferriprotoporphirin IX (36, 37). In other works, it was suggested that ferriprotoporphirin IX or other closely related porphyrin might be incorporated to the molecule of bovine (38) and hog TPO (39, 40). The probable localization in the TPO structure of proximal histidine (linked to the iron centre of the heme) and distal histidine (situated close to the peroxide binding pocket) was firstly proposed by Kimura and Ikeda-Saito (41). They indicated His407 or His414 as the candidates for the proximal and His494 or His586 as the probable distal ligand. More recently, His494 (42, 43) and His239 (43) were pointed out as possible proximal and distal histidine residues, respectively. It is hypothesized that Glu399 and Asn238 are bound covalently to the heme prosthetic group through ester linkage (43, reviewed in Ref. 44).

The group of JL Franc (45) investigated the role of the heme moiety insertion in the exit of TPO from the endoplasmic reticulum. The treatment of TPO expressing CHO cells with succinyl acetone (SA), an inhibitor of heme biosynthesis, resulted in significant reduction in peroxidase activity at the cell surface and cell surface expression of TPO. On the contrary, supplementation of the culture medium with precursors of heme biosynthesis improved the TPO delivery to the cell membrane and TPO activity in this compartment. Thus, it seems that heme incorporation plays an essential role in the intracellular trafficking of

TPO. In the same study, the role of H_2O_2 in heme binding to the TPO molecule was investigated (45). It was demonstrated, both in TPO-CHO cells and in porcine thyroid primoculture, that heme is autocatalytically modified in the presence of H_2O_2 and subsequently covalently linked to the TPO protein. This process starts inside the cell, where H_2O_2 is probably generated by electron transfer reactions (in CHO and thyroid cells), and it is continued at the cell surface, where H_2O_2 is produced by DUOX1 and DUOX2 (in thyroid cells). Unlike in thyrocytes, the surface H_2O_2-generating system is not present in TPO-CHO cells, therefore, H_2O_2 supplementation significantly improves the autocatalytic covalent attachment of heme in this cell line (45).

During maturation TPO undergoes proteolytic trimming in the N-terminal part. The group of JL Franc (27) showed that N-sequence of the TPO molecule, affinity purified from the human thyroid tissue, begins at Thr109. They suggested that the sequence running from the signal peptide cleavage site to Thr109 is a propeptide. Many such sequences encoded as N-terminal or C-terminal extensions have been identified to function as intramolecular chaperones (reviewed in Ref. 46). Intramolecular chaperones are divided into two categories depending on their role in protein folding. The intramolecular chaperones classified to the type I assist tertiary structure formation and they are mostly located at the N-terminus of the protein. Type II intramolecular chaperones are more responsible for quaternary structure formation to guide the functional protein complex assembly rather than directly employed in tertiary structure generation. These chaperones are predominantly produced as the C-terminal sequence extensions (reviewed in Ref. 46). To test the role of TPO propeptide in protein maturation, a series of experiments on recombinant human TPO were conducted (27). Deletion of propeptide (Cys15-Lys108) resulted in reduction in proportion of the folded TPO form. Because the signal peptide cleavage site is not known the shorter propeptide was also deleted (Lys27-Lys108), however, the results were identical. Apart from affecting proper folding, the propeptide deletion caused complete inhibition of TPO surface expression and thyroperoxidase activity. Moreover, it was deduced that the N-proteolysis in TPO-CHO cells occurs after its transit through the Golgi apparatus but before it reaches the cellular surface. Propeptide cleavage is probably carried out by some protein convertase (PPC). Site directed mutagenesis of the potential motifs in the TPO sequence theoretically recognized by known PPC enzymes did not inhibit the whole propeptide removal in CHO, K562, and PC Cl3 cell lines. Therefore, in human thyroid gland the cleavage process probably differs from that occurring in these three cell lines (27).

It seems that apart from myeloperoxidase (reviewed in Ref. 47), TPO is the second member of mammalian peroxidase family that forms homodimers. Firstly, it was observed that anti-TPO antibodies recognized a protein of about 200 kDa, approximately twice the size of human TPO, when TPO isolated from human gland (48-51) or expressed in CHO cells (52, 53) was separated by SDS-PAGE under non-reducing conditions. More recently, TPO dimers formation was demonstrated using co-immunoprecipitation and ELISA binding studies in TPO produced in CHO cells (54). Interestingly, homodimers were resistant to denaturation with conventional reducing agents (54). Additionally, dimerisation process was sensitive to thionamide treatment that is used to cure Graves' disease and other forms of hyperthyroidism (54). Overall, further studies are needed to explain how and where in the thyroid cell the dimer of TPO is formed.

TPO INTRACELLULAR TRAFFICKING AND TARGETING

TPO is first synthesized within the endoplasmic reticulum and then intracellularly transported by the secretory pathway to the cell surface. The fraction of total cellular TPO protein residing at the plasma membrane in primary porcine thyrocytes is limited and estimated at about 30% of total TPO in seven-day culture (55) or even less (about 5% of total TPO) in eighteen-day culture (56). However, preliminary studies on the rat TPO distribution in rat thyroid tissue homogenate showed that TPO is well expressed at the plasma membrane (57). Further studies on recombinant TPO expressed in various cell lines resulted in different conclusions concerning the transport efficiency. Fayadat and collaborators (26) stated that only about 2% of full-length TPO reaches the surface of stably transfected CHO cells, while most TPO molecules stays in the endoplasmic reticulum. Zhang and Arvan (31) stably transfected TPO to MDCK and PC Cl3 cells and transiently to CHO cells. After isolation and expansion of individual clones (similarly as in Ref. 26) they observed by immunofluorescence that no more than 10% of total TPO is at the surface on MDCK cells and even more in CHO and PC Cl3 cells. It was hypothesized that the lower surface expression of TPO described by Fayadat et al. (26) may result from the method used for surface protein isolation, namely surface protein biotynylation, that seems to underestimate TPO localization in plasma membrane in comparison with immunofluorescence assay (31). Furthermore, the results obtained for the same TPO construct may significantly differ depending on the cell line, in which the recombinant protein is produced (31). These differences may be a consequence of heterogeneous cell surface expression that occurs even within a clonal population in which all cells express TPO intracellularly (31). This phenomenon may be explained by the hypothesis of McLachlan and Rapoport (10) that high concentration of cell-anchored TPO can be toxic to cultured cells and influence a negative selective pressure.

As it has been already underlined, only a part of intracellular TPO goes to the membrane. It was shown that at the ER level TPO expressed in CHO cells is degraded via two different pathways, depending on its folding state (34). The completely unfolded form is degraded by the proteasome, whereas the partially folded form is degraded by another proteolytic system (34). More recent studies showed that a plasminogen (Pl)-like protease may be responsible for nonproteasomal TPO

degradation at the ER level both in CHO cells and in the thyroid follicles (58). Interestingly, the same enzyme was earlier identified as a thyroglobulin (Tg) degrading agent in the thyroid (59), therefore, it was hypothesized that Pl-like protease contributes to regulation of the pool and/or trafficking of TPO and Tg (58).

During processing TPO interacts with several molecular chaperones. In CHO cells co-transfected with TPO, calnexin (CNX), and calreticulin (CRT), these two chaperones bind to TPO in the ER and these interactions are required for the correct folding of TPO to occur (34). Inhibiting the association between CNX or CRT and TPO using castanospermine (CST) resulted in great increase in the degradation of TPO molecules. CST blocks the trimming of the three glucoses from the core oligosaccharide and the subsequent interaction between CNX or CRT and the glycoprotein substrate (34). Thus, CST treatment caused reduction in the TPO half-life by about 7 times, and consequently, resulted in degradation of TPO via the proteasome pathway and decreased TPO expression at the cell surface (34). It was established that CNX and CRT form a complex with ERp57 and together are key molecular players in the quality control of newly synthesized glycoproteins (reviewed in Ref. 60). ERp57, the multifunctional thiol oxidoreductase, is responsible for the rearrangement of disulfide bonds (reviewed in Ref. 60). Overexpression of CNX and ERp57 in TPO-CHO cells increased the quantity of TPO obtained in pulse-chased experiments by stabilizing the protein immediately after its synthesis (35). In the same study, the role of other molecular chaperone, BiP, in the maturation of TPO was also examined (35). It seems that BiP and calnexin have opposite effects on the folding process of the TPO molecule in CHO cells (35). These two chaperones compete for the binding to newly synthesized TPO and drive it to either the maturation (CNX) or degradation (BiP) pathway (35). Wang et al. (61) isolated a complex of TPO with EF-hand binding protein (EFP1) by co-immunoprecipitation from human thyrocytes in primary culture. They hypothesized that EFP1 may play a role as a chaperone-like protein in TPO and DUOXs processing, however, more studies are needed to confirm this assumption.

PHYSIOLOGICAL FUNCTION IN THYROID HORMONE SYNTHESIS

Thyroid peroxidase is a key enzyme responsible for the thyroid hormone biosynthesis. This process occurs at the apical membrane-colloid interface of thyrocytes and requires the presence of three components: iodide, H_2O_2, and thyroglobulin. Sodium iodide symporter (NIS) mediates active transport of iodide into the follicular thyroid cell. Inside the cell, iodide is driven through the apical membrane to the follicular lumen by pendrin (62) and other unknown systems (reviewed in Ref. 63). H_2O_2 is generated by DUOX1 and DUOX2 (reviewed in Ref. 64, 65) and it is rapidly used by TPO to oxidize iodide resulting in covalent binding to the tyrosyl residues of Tg. This step generates monoiodotyrosine (MIT) and diiodotyrosine (DIT). Then the coupling of two appropriate iodotyrosine residues occurs. Two DIT will form thyroxine (T4), whereas one MIT and one DIT will form triiodothyronine (T3). The coupling reaction is catalyzed by TPO in the presence of H_2O_2 and takes place provided that the iodotyrosine residues are properly spaced to each other in Tg molecule. The cleavage reaction in Tg releases thyroid hormones into the circulation (reviewed in Ref. 2).

Although H_2O_2 at low levels may have physiological function inside the cells, e.g., in signal transduction, in excess, it might induce mutagenesis, carcinogenesis, or apoptosis (reviewed in Ref. 64). Therefore, the H_2O_2-generating system is strictly limited to the microvilli of the apex of follicular cells to protect the interior of the cells from this reactive oxygen species. Song et al. (64) hypothesized that it would be beneficial if the proteins constituting the H_2O_2-generating system formed a complex with TPO, called thyroxisome, to increase working efficiency and minimize H_2O_2 leakage.

It was shown by co-immunoprecipitation that TPO and DUOXs associate with each other in primary culture of human thyrocytes (61). More detailed studies, using the total lysates and plasma membranes from human primocultured thyrocytes and co-immunoprecipitation assay, demonstrated that DUOXs and TPO locate closely in the plasma membrane (66). The structure of human DUOX protein has the long N-terminal peroxidase-like extracellular ectodomain that shares 43% similarity with TPO (reviewed in Ref. 64, 65). Therefore, it was proposed that this region might form a heterodimer with thyroid peroxidase mediated through cysteine disulfide bounds (64, 66). The results, obtained using truncated DUOXs co-expressed with TPO in COS-7 cells, support this hypothesis (66). It was suggested that there is no physiological association between DUOX and TPO inside the thyrocytes because the TPO expression is not necessary to bring DUOX enzymes to the cell surface (66). Now it is only well documented that DUOX 1 and DUOX 2 need the presence of their maturation factors (called DUOXA 1 and DUOXA2, respectively) to exit from the endoplasmic reticulum and to reach the apical plasma membrane (reviewed in Ref. 65). However, in DUOX2-expressing HEK293 cells transfected with TPO and in the absence of DUOXA2 at the same time, there was observed a slight stimulation of DUOX2 activity that was not related to an increase in the DUOX2 protein content or to a change of the glycosylation status (67). The interpretation was that the resting form of DUOX2 located in the endoplasmic reticulum may interact with TPO (67). Nevertheless, even if the complex DUOX-TPO is formed inside the cell it seems that it is inactive to protect the cell interior from H_2O_2.

The analysis of the physiological regulation of the DUOX-TPO complex formation was conducted on the follicle freshly prepared from human thyroid (66). After treating open follicles with different stimuli, the plasma membranes were isolated followed by the co-immunoprecipitation of DUOX and TPO. It was observed that the association was increased by the activation of the Gq-phospholipase C-Ca^{2+}-protein kinase C pathway and down-regulated through the Gs-cAMP-protein kinase A pathway (66).

Recently there have been conducted detailed studies on the functional consequences of DUOX-TPO interaction at the plasma membrane of transfected HEK293 cells (68). It was observed that both DUOX1 and DUOX2 are retro-inhibited by H_2O_2 that they have produced at the cell surface. DUOXs appear to be sensitive to oxidative modifications that cause conformational changes leading to the decrease in these enzymes activity. The role of TPO is to protect DUOXs from this inhibition probably by a catalase-like activity of the TPO protein. This activity of thyroid peroxidase was dependent on the interaction with DUOX2. Site directed mutagenesis of the N-terminal cysteine residues in DUOX2 resulted in decrease in the H_2O_2 generating activity of this enzyme. Therefore, integrity of the peroxidase-like domain of DUOX2 seems to be important for the protective role of TPO. The DUOX2-TPO interaction might cause conformational changes of the TPO protein, and as a consequence, stimulate the catalase activity of the latter (68). The protective effect of TPO on DUOX1 suppression by H_2O_2 was also observed (68). H_2O_2 has inactivating effect not only on DUOX enzymes but also on TPO. Thyroid peroxidase processes H_2O_2 inducing its own oxidation and consequently its own inactivation (68). In the absence of the reducing substrate (iodide) TPO may form protein radicals that are enzymatically inactive (69). It was hypothesized that radical-damaged TPO may be assigned as foreign by the immune system and subsequently trigger autoimmunological response (69).

CONCLUSIONS

Although our knowledge on human thyroid peroxidase is extensive, there are still some questions that await answer. We know very little of dimer formation process. The mechanism of interaction between thyroid peroxidase and thyroglobulin in thyroid hormone synthesis is also unclear. Furthermore, the problem with solving of the three-dimensional structure by crystallization causes difficulties, e.g., in thorough understanding of the interactions between TPO and autoantibodies.

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