CLINICAL STUDY

Negative correlation between thyroperoxidase and dual oxidase H$_2$O$_2$-generating activities in thyroid nodular lesions

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Abstract

Iodine incorporation into thyroglobulin is dependent on the activities of both thyroperoxidase (TPO) and thyroid dual oxidase 2 (DuOx2). Although TPO expression is decreased in some thyroid nodular lesions, DuOx1 and 2 mRNA expressions are maintained, but DuOx H$_2$O$_2$-generating activity has never been evaluated in such tumors. Our goal was to determine DuOx activity in hypofunctioning lesions of the thyroid. We evaluated H$_2$O$_2$ generation by DuOx in 12 paranodular to cold nodule samples, 17 non-toxic multinodular goiters (MNG; 33 samples), 3 papillary carcinomas (PC; 4 samples), 3 follicular carcinomas (FC; 4 samples), and 10 follicular adenomas. DuOx activity was detected in all paranodular tissues (121±23 nmol H$_2$O$_2$/h per mg protein), but was undetectable (<1 nmol H$_2$O$_2$ generated) in all PC, two out of four FC samples and seven out of ten adenomas. In 11 MNG at least two different areas of the goiter have been evaluated, and in 5 of these goiters one of the samples had DuOx activity below the limit of detection. The coefficient of variation in MNG samples ranged from 11.3 to 57.2%. Interestingly, in all the adenomas studied, TPO activity (486±142 U/g protein, n=8) was well within the range found in paranodular tissues (414±116 U/g protein, n=3). We found a significant negative correlation between DuOx and TPO activities, suggesting that these enzymes are regulated in opposite directions, at least in thyroid tumors.

Introduction

The concentration of iodine in the thyroid gland depends on the presence of several proteins that are necessary for iodide uptake through the basolateral membrane of thyrocytes and its incorporation into the acceptor protein thyroglobulin (Tg) at the apical surface of these cells (1, 2). The sodium–iodide symporter is responsible for thyroid cell basolateral iodide uptake against an electrochemical gradient. At the apical pole of thyrocytes, intracellular iodide is transported through pendrin (Pendred syndrome gene PDS) (3) into the follicular lumen and then incorporated into Tg. Iodide oxidation and organification occur at the apical surface of the follicular cell and these reactions are catalyzed by thyroperoxidase (TPO) in the presence of hydrogen peroxide. Thus, thyroid iodide organification depends on TPO activity, which is modulated by the concentration of Tg, iodide and hydrogen peroxide (1, 2). In the presence of sufficient amounts of iodine, the limiting step for thyroid hormone biosynthesis is the availability of H$_2$O$_2$, which is generated by thyroid dual oxidase (DuOx) (4).

Hypofunctioning tumoral lesions of the thyroid gland are characterized by impaired iodine uptake and/or organification. Iodine incorporation into Tg is dependent on the activities of both TPO and DuOx. Although TPO expression is decreased in some thyroid nodular lesions, DuOx1 and 2 mRNA expressions are maintained (5–7). DuOx H$_2$O$_2$-generating activity depends not only on enzyme expression but also mainly on its maturation and normal subcellular distribution in thyrocytes, as recently demonstrated (8). Thus, the expressions of DuOx mRNA and proteins do not necessarily imply normal hydrogen peroxide-generating activity, so our goal was to determine DuOx H$_2$O$_2$-generating activity in hypofunctioning lesions of the thyroid. We evaluated NADPH/calcium-dependent H$_2$O$_2$ generation by DuOx in 12 paranodular to cold nodule samples (PN), 17 non-toxic multinodular goiters (MNG), 3 papillary carcinomas (PC), 3 follicular carcinomas (FC), and 10 follicular adenomas (AD). Interestingly, the H$_2$O$_2$-generating activity was undetectable in the majority of samples of thyroid carcinomas and adenomas. Also, we show herein
that a significant negative correlation between TPO- and $H_2O_2$-generation activities does occur in human thyroids.

**Materials and methods**

**Materials**

NADPH and lyophilized horseradish peroxidase (HRP, grade 1) were purchased from Boehringer; BSA, Scopoletin and flavin adenine dinucleotide (FAD) were obtained from Sigma Chemical Co.

**Patients and thyroid tissue samples**

The study has been approved by the Institutional Committee for Ethics in Human Research. Thyroid tissue samples were obtained during elective thyroidectomy. Samples from PN surrounding cold thyroid nodules ($n=12$, females, median age $=44.5$ years) were used as control. We have also evaluated 17 patients with MNG ($14$ females, $3$ males, median age $=53$ years); $3$ FC (2 females, one at stage T1N1Mx and one at T3N1Mx and 1 male at stage T1N1Mx, median age $=26$ years); $3$ FC (all females, two at stage T3N1Mx and one at T2N1Mx, median age $=51$ years); and $10$ AD (9 females, 1 male, median age $=46$ years). In $11$ MNG, 2–5 different areas of the goiter have been processed for enzymatic activity. In one of the papillary and one of the FC, two different areas have been analyzed, which correspond to carcinoma areas, as confirmed by anatomopathological diagnosis. Patients received no medication before surgery. Thyroid tissue samples were either immediately processed for $H_2O_2$-generation measurements or stored at $-70\degree{C}$ for later TPO extraction.

**Thyroid sample processing**

For NADPH oxidase (DuOx) activity, fresh thyroid tissue samples (1 g) were cleaned from fibrous tissue or hemorrhagic areas, minced, and homogenized in 50 mM sodium phosphate buffer (pH 7.2) containing 0.25 M sucrose, 0.5 mM dithiothreitol, and 1 mM EGTA, using an Ultra-Turrax (IKA, Staufen, Germany). The homogenate was centrifuged at 100 000 g for 1 h at 4 °C, and the pellet was resuspended in 2 ml digitonin (1%, w/v). The mixture was incubated at 4 °C for 24 h and then centrifuged at 100 000 g for 1 h, 4 °C. The supernatant containing solubilized TPO was used for the iodide-oxidation assays, as previously described (11–13).

Protein concentrations were measured by the method of Bradford (14). The particulate fractions used to measure DuOx activity were incubated with 1 M NaOH (30 min, 20 °C) to dissolve the particulate before protein determination.

**Ca$^{2+}$- and NADPH-dependent $H_2O_2$-generating activity – DuOx activity**

$H_2O_2$ formation was measured as previously described (9, 10). We incubated samples of the thyroid particulate fractions at 30 °C, in 1 ml 170 mM sodium phosphate buffer (pH 7.4) containing 1 mM sodium azide, 1 mM EGTA, 1 µM FAD, and 1.5 mM CaCl$_2$. Adding 0.2 mM NADPH started the reaction; aliquots of 100 µl were collected at intervals up to 20 min, and mixed with 10 µl 3 M HCl to stop the reaction and destroy the remaining NADPH. Initial rates of $H_2O_2$ formation were determined from eight aliquots of each assay by following the decrease in 0.4 µM scopoletin fluorescence in the presence of HRP (0.5 µg/ml) in 200 mM phosphate buffer (pH 7.8) in a Hitachi spectrofluorimeter (F 4000). The excitation and emission wavelengths were 360 and 460 nm respectively. Specific activities were expressed per mg protein (nmol $H_2O_2$/h per mg protein).

**TPO iodide-oxidation activity**

Thyroid peroxidase iodide-oxidation assays were performed using 12 mM KI in 50 mM phosphate buffer (pH 7.4), and glucose–glucose oxidase as the hydrogen peroxide ($H_2O_2$)-generating system, as previously described (11–13). The increase in absorbency at 353 nm ($\Delta A_{353}$) was followed for 4 min on a U-3300 Hitachi double beam spectrophotometer. The TPO activity was estimated from the $\Delta A_{353}$/min determined from the linear portion of the reaction curve. One unit of iodide oxidation activity is defined as $\Delta A_{353}$/min (U) $=1.0$, and activity was related to the protein concentration in the enzyme preparation (U/g protein).

**Statistical analysis**

Enzymatic values were analyzed by non-parametric one-way ANOVA (Kruskal–Wallis test) followed by Dunn’s multiple comparison test.

Analysis of correlation between DuOx and TPO activities has been done by the Spearman test.
Results

DuOx activity was detected in all 12 PN (121 ± 23 nmol H₂O₂/h per mg protein) tissues, but was undetectable (<1 nmol H₂O₂ generated) in all 4 PC samples, 2 out of 4 FC samples, and 7 out of 10 AD (Fig. 1A). One FC with undetectable activity was at the same stage as the other with normal DuOx activity (both at T3NxMx), suggesting that tumor stage might not be the only factor involved in the loss of hydrogen peroxide generation. The activity was undetectable in at least one tissue sample from 7 out of 17 non-toxic MNG patients. In 11 MNG, at least two different areas of the goiter have been evaluated with a coefficient of variability for each goiter ranging from 11.3 to 57.2%, values that do not differ from the variability found in PN tissue samples that were obtained from different patients (coefficient of variation, CV = 66%). In 5 out of these 11 MNG with more than one sample analyzed, the activity was below the limit of detection in at least one of the samples. Although previous reports (5, 6) demonstrate a normal or even high DuOx2 mRNA and protein expression in hypofunctioning thyroid lesions including carcinomas, DuOx H₂O₂-generating activity was undetectable in the majority of thyroid tumors analyzed herein.

TPO activity was evaluated in some of these nodules, showing a large variability in thyroid tumor samples, as previously reported (Fig. 1B) (12). Interestingly, while DuOx activity was undetectable in seven adenomas studied, TPO (486 ± 142 U/g protein, n = 8) was well within the range found in PN tissues (414 ± 116 U/g protein, n = 3; Fig. 1B) in all of them. Also, in two PC with undetectable DuOx activities, TPO was in the normal range. Both TPO and DuOx activities were undetectable in only one of the PC evaluated. On the other hand, in the two FC with normal DuOx activities, TPO was undetectable (one at T3NxMx and the other at T3NxMx). In contrast, in the carcinoma with normal TPO an undetectable DuOx activity was found although staging corresponds to T3NxMx, such as the other FC with undetectable TPO. In only two samples of MNG both DuOx and TPO activities were absent.

In the samples in which both DuOx and TPO activities were measurable, a significant negative correlation (Fig. 2B) was found, suggesting that these enzyme activities are regulated in the opposite direction in human thyroids, at least during tumorigenesis.
Discussion

After the cloning of DuOx1 and 2 cDNAs, some studies have contributed to a better understanding of the regulation of their expression in the thyroid gland and other tissues (5–7, 10, 15–17). A significant positive correlation between DuOx1 and 2 gene expressions has been demonstrated, although the role of DuOx1 in thyroid H2O2 generation associated to hormonogenesis remains to be elucidated (5). On the other hand, DuOx2 participation on hydrogen peroxide generation associated with TPO has been confirmed by original reports describing homozygous mutations in the gene (18) and biochemical studies demonstrating impaired calcium/NADPH-dependent H2O2 generation as the cause of dyshormonogenetic goiters (19). However, only recently functional studies on the role of either DuOx1 or DuOx2 have turned out to be possible, after the identification of two maturation factors, DuOxA1 and DuOxA2, which are necessary for the correct processing of either DuOx1 or 2 respectively (8).

Although some reports have shown normal or even high DuOx2 mRNA expression in human thyroid nodules (5, 6), and normal DuOx protein expression in several tumoral lesions of the thyroid gland (7), to date no evaluation of calcium/NADPH hydrogen peroxide generation has been performed in human sporadic goiters. We show herein that in fact H2O2 generation is impaired in several hypofunctioning thyroid nodules, although DuOx protein expression might be normal, as previously reported (7). A great variability in hydrogen peroxide-generation ability has been detected in MNG, although the activity was well within the range found in paranodular tissues in the majority of MNG samples evaluated. Interestingly, in AD and thyroid carcinomas, H2O2-generation ability by DuOx was decreased or even abolished; however, our data do not exclude the possibility that increased H2O2 production might occur during early stages of tumorgenesis. Our data are consistent with the idea that hydrogen peroxide production associated to hormonogenesis corresponds to a differentiation marker that might be downregulated during tumor progression. Further studies are needed in order to evaluate the expression of DuOx maturation factors (DuOxA) in thyroid nodules, since the lack of these factors might indeed lead to decreased H2O2 production, notwithstanding normal mRNA and protein DuOx expressions.

TPO expression is tightly regulated by thyrotropin (TSH), but DuOx seems not to be so dependent on the TSH pathway in mouse thyroids at least, as recently demonstrated (20). In contrast, calcium/NADPH-dependent H2O2 production was shown to be positively regulated by TSH and forskolin in porcine (21) and human (22) thyroids, which correspond to a regulation pattern similar to the one described for TPO expression. In the present study we report a significant negative correlation between TPO and DuOx activities, and these data are difficult to explain in light of our current knowledge about the regulation of their gene expressions. The main regulator of thyroid hydrogen peroxide generation seems to be the intracellular iodine content, depending on the species studied (10, 23–25). However, decreased hydrogen peroxide generation in hypofunctioning lesions might not be explained by increased iodine content in these lesions, so another mechanism seems to be involved in the downregulation of H2O2 generation. In contrast, in lesions with measurable TPO and H2O2, a higher TPO activity might lead to increased iodine content and thus could explain the decreased H2O2 production, as suggested by the negative correlation found. These findings suggest that DuOx regulation by iodine might correspond to a tight mechanism of possible physiological importance.

We conclude that calcium/NADPH-dependent H2O2 generation is downregulated in the majority of human hypofunctioning lesions, such as AD and differentiated thyroid carcinomas. Also, in thyroid samples in which both TPO and hydrogen peroxide generations were measurable, we found that the higher the TPO activity, the lower the hydrogen peroxide generation. The opposite regulation of these enzymes involved in thyroid iodine organisation might be secondary to a tight modulation of TPO expression and an as yet undefined mechanism of regulation of hydrogen peroxide generation.

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