Minireview

Dual oxidase, hydrogen peroxide and thyroid diseases

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Abstract

The thyroid gland is a unique endocrine organ that requires hydrogen peroxide (H_2O_2) for thyroid hormone formation. The molecule for H_2O_2 production in the thyroid gland has been known as dual oxidase 2 (DUOX2). Recently, NADPH oxidase 4 (NOX4), a homolog of the NOX family, was added as a new intracellular source of reactive oxygen species (ROS) in the human thyroid gland. This review focuses on the recent progress of the DUOX system and its possible contribution to human thyroid diseases. Also, we discuss human thyroid diseases related to abnormal H_2O_2 generation. The DUOX molecule contains peroxidase-like and NADPH oxidase-like domains. Human thyroid gland also contains DUOX1 that shares 83% similarity with the DUOX2 gene. However, thyroid DUOX1 protein appears to play a minor role in H_2O_2 production. DUOX proteins require DUOX maturation or activation factors (DUOXA1 or 2) for proper translocation of DUOX from the endoplasmic reticulum to the apical plasma membrane, where H_2O_2 production takes place. Thyroid cells contain antioxidants to protect cells from the H_2O_2-mediated oxidative damage. Loss of this balance may result in thyroid cell dysfunction and thyroid diseases. Mutation of either DUOX2 or DUOXA2 gene is a newly recognized cause of hypothyroidism due to insufficient H_2O_2 production. Papillary thyroid carcinoma, the most common thyroid cancer, is closely linked to the increased ROS production by NOX4. Hashimoto's thyroiditis, a common autoimmune thyroid disease in women, becomes conspicuous when iodide intake increases. This phenomenon may be explained by the abnormality of iodide-induced H_2O_2 or other ROS in susceptible individuals. Discovery of DUOX proteins and NOX4 provides us with valuable tools for a better understanding of pathophysiology of prevalent thyroid diseases.

Keywords: dual oxidase, hydrogen peroxide, congenital hypothyroidism, Hashimoto's thyroiditis, papillary thyroid cancer

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Introduction

Hydrogen peroxide (H_2O_2) is essential for thyroid peroxidase (TPO)-catalyzed thyroid hormone formation. The ability of the thyroid to generate H_2O_2 was reported in 1971.[1,2] Björkman *et al.*[3,4] demonstrated that H_2O_2 in the thyroid gland was produced by NADPH oxidase located at the apical plasma membrane. Virion *et al.*[5] proposed that H_2O_2 generation by the thyroid NADPH oxidase does not involve the intermediate superoxide (O_2^-) as an electron acceptor. NADPH oxidase-mediated H_2O_2 generation in the thyroid gland requires Ca^{2+} to be fully active.[4,6–9] This enzyme system has a membrane-bound flavoprotein using flavin adenine dinucleotide (FAD) as a co-factor.[10,11] In 1999, Dupuy *et al.*[12] first reported the cloning of the pig and human $p138^{Tox}$ flavoproteins consisting of 1207 (porcine) and 1210 (human) amino acids. In 2000, De Deken *et al.* cloned the entire two human thyroid cDNAs encoding new NADPH oxidase members by screening cDNA libraries using a probe of $gp91^{phox}$/NOX2, a well-defined NADPH oxidase in phagocytes. The two genes were initially called thyroid oxidase (THOX) 1 and 2. THOX 2 turned out to be the full-length version of $p138^{Tox}$ flavoprotein.[13] Now, these two oxidases are named as DUOX1 and DUOX2. The name of DUOX (DUal OXidase) is derived from the protein structure analysis of NADPH oxidase family of *Caenorhabditis elegans*.[14] Thyroid diseases derived from the DUOX system have not been well recognized with the exception of the congenital hypothyroidism due to mutations in the DUOX2 gene.[15–21] Weyemi *et al.*[22] recently reported a new intracellular reactive oxygen species (ROS) generating system (NADPH oxidase 4 [NOX4]) in the human thyroid gland.

Thyroid hormone biosynthesis

Figure 1 shows the schema of thyroid hormone biosynthesis. Iodide is actively transported into thyrocytes by a sodium/iodide symporter (NIS) on the basolateral membrane and to the follicular lumen by, in part, pendrin (PDS/SLC26A4) at

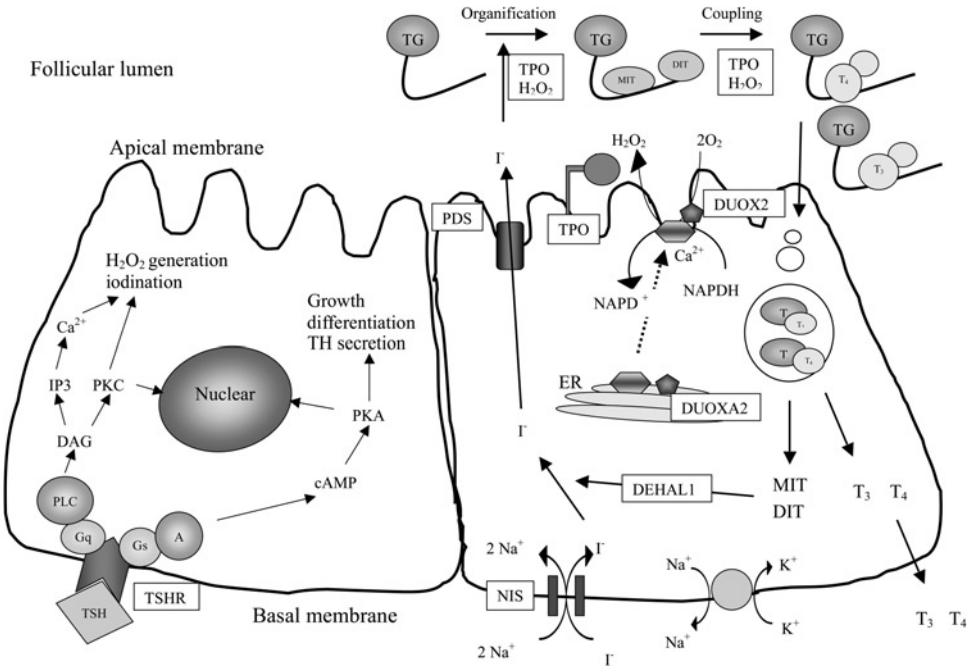

Figure 1 Thyroid hormone biosynthesis, secretion and major signaling pathways in thyrocytes. AC, adenyl cyclase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; DEHAL1, dehalogenase 1; DIT, diiodotyrosine; DUOX, dual oxidase; DUOXA, dual oxidase maturation factor; IP3, inositol trisphosphate; MIT, monoiodotyrosine; NIS, sodium-iodide symporter; PDS, pendrin; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; T3, triiodothyronine; T4, thyroxine; TG, thyroglobulin; TPO, thyroid peroxidase; TSH, thyrotropin; TSHR, thyrotropin receptor

the apical membrane. Iodide is rapidly oxidized by TPO in the presence of H_2O_2 resulting in covalent binding to the tyrosyl residues of thyroglobulin (Tg) on the luminal side of the apical membrane. This step produces monoiodotyrosine (MIT) and diiodotyrosine (DIT). Then only properly spaced MIT and DIT in Tg participate in the coupling reactions to form thyroxine (T_4) and triiodothyronine (T_3); this reaction is also catalyzed by TPO with H_2O_2. The source of thyroid H_2O_2 is DUOX2 expressed in the apical plasma membrane coordinated with DUOXA2. Thyroid hormones are released into the circulation after digestion of Tg. MIT and DIT are deiodinated by an iodotyrosine dehalogenase 1 (DEHAL1).[23] Thyroid hormone formation is predominantly regulated by thyrotropin (TSH). The binding of TSH to the TSH receptor activates both Gs and Gq proteins. The former activates the growth regulation, differentiation and thyroid hormone secretion, whereas the latter activates H_2O_2 generation and iodide binding to protein through the phospholipase C-dependent inositol phosphate Ca^{2+}/diacylglycerol pathway.[24]

DUOX genes and proteins

DUOXs' structure

Human Duox1 and Duox2 genes are located 16 kb apart on the chromosome 15q15.3. These genes have opposite transcriptional orientations.[25] The Duox1 gene spans 36 kb and contains 35 exons; the first two exons are non-coding. The Duox2 gene spans about 22 kb composed of 34 exons, and the first exon is non-coding.[12,13] Human DUOX1 and DUOX2 proteins have 1551 amino acids and 1548 amino acids, respectively, for open reading frame. DUOX1 and DUOX2 show 83% similarity in their DNA sequences.[13] However, the DUOX1 and DUOX2 promoters are different. The human DUOX1 promoter has GC-rich three putative SP1-binding sites. The human DUOX2 promoter has neither a TATA box nor an SP1-binding element.[16] The DUOXs are glycoproteins containing seven transmembrane helices, an extracellular peroxidase-like domain, a long intracellular loop with two EF-hand motifs and a NOX2-like domain starting from around the second transmembrane helix to the C-terminal, in which two critical binding cavities for FAD and NADPH are present (Figure 2).[12,13] The peroxidase-like domain has 43% similarity to TPO. Whether the peroxidase-like domain in humans exhibits peroxidase activity is controversial. Both detection of peroxidase activity[14,26] and absence of peroxidase activity[27] have been reported. The DUOX proteins have two EF-hands, calcium-binding sites on the first intracellular loop, suggesting an important regulatory role of Ca^{2+} for the DUOX molecule. Indeed, an enhanced H_2O_2 generation by Ca^{2+} has been shown from the DUOX2 in transfection experiments.[28–30] Rigutto *et al.*[30] also confirmed a significant contribution of the EF-hands to H_2O_2 production, since the introduction of mutation to glutamate residues of EF-hands did not stimulate H_2O_2 production by ionomycine. DUOX1 and DUOX2 have 53% and 47% similarity to gp91^{phox}/NOX2, respectively.[13] The NOX2-like domain of the DUOX molecule retains necessary structures and functions of phagocyte NOX2 for electron transfer from NADPH to FAD, heme and extracellular molecular oxygen.[31,32]

Tissue distribution of the DUOX1 and 2

In the thyroid gland, the DUOX2 mRNA expression is 1.5–5 times more abundant than that of DUOX1.[25] The DUOXs are located at the apical membrane of thyrocytes and

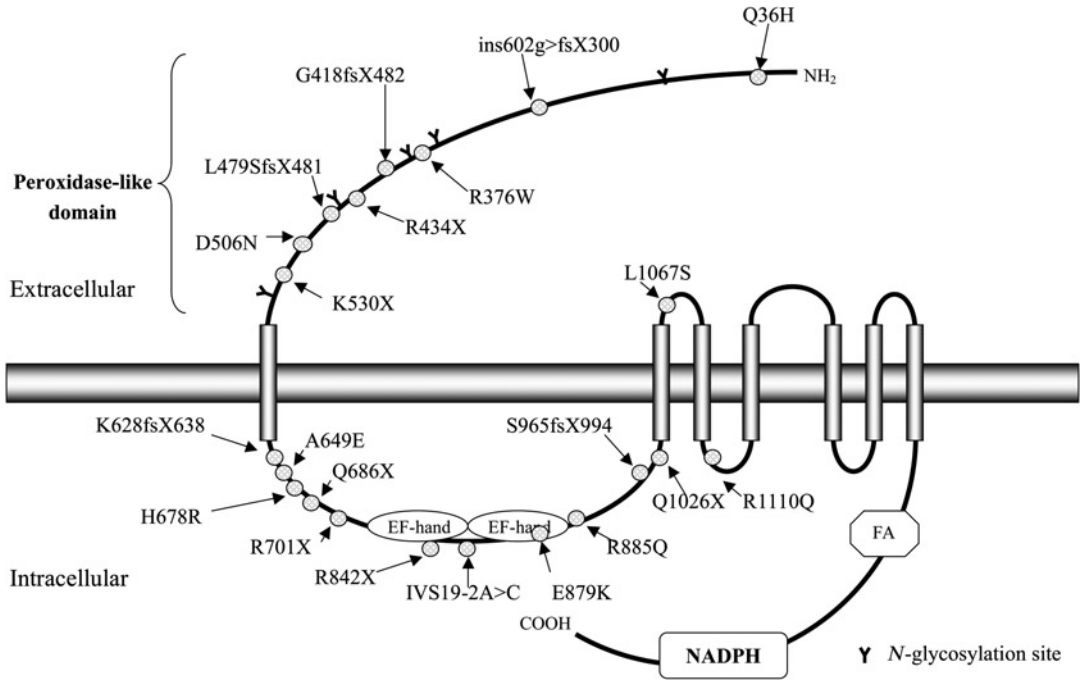

Figure 2 Structural model of DUOX2 protein and sites of reported mutations. Mutation sites at the amino acid level are indicated by small closed circles. Arrows explain changes in amino acids resulting from the mutations. The peroxidase-like domain is located in the N-terminal extracellular portion. Two EF-hands for Ca^{2+}-binding sites are on the first long intracellular loop. FAD and NADPH binding cavities are on the intracellular C-terminal portion. FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate

co-localized with TPO.[13] The DUOX1 and DUOX2 expressions are not restricted to the thyroid gland.[14] DUOX1 is present in the prostate, testis, placenta, heart, kidney, brain, pancreas and skin. DUOX2 is found in the salivary gland, stomach, duodenum, colon, rectum, pancreas and testis.[14,33–35] In human airway epithelial cells, both DUOX1 and 2 are expressed.[34,36–38]

Requirement of DUOX activation factors for DUOX protein maturation and transition to the plasma membrane

Only fully glycosylated DUOX2 is transported to the plasma membrane and generates H_2O_2, whereas partially glycosylated DUOX2 remains in the endoplasmic reticulum (ER) that generates O_2^-.[29,39–41] Grasberger *et al.*[39] identified the DUOX maturation factors (DUOXA1 and DUOXA2) that enable the DUOX proteins to translocate to the targeting membrane and display a full enzymatic activity of DUOXs. Only the combination of the DUOXs with their corresponding DUOXAs makes the functional unit for proper folding of DUOXs to exit the ER.[40] The DUOXA genes are located within the 16 kb intergenic region between DUOX1 and DUOX2 genes in a tail-to-tail orientation to each other. The DUOXA2 protein consists of 320 amino acids, six exons and five membrane-integral regions with an extended extracellular loop between second and third transmembrane domains, where the three *N*-glycosylation sites are present. The DUOXA1 gene is located adjacent to DUOXA2 and extends into the promoter region of DUOX1.[39] The DUOXA2 mRNA is more abundantly expressed in the thyroid gland than in the salivary gland. The DUOXA1 mRNA is also predominantly distributed in the thyroid gland, with a lower level in the esophagus.[39] The DUOXA1 is also expressed in human respiratory epithelial cells.[42]

The DUOXA1 has four transcript variants: DUOXA1-α, -β, -γ and -∂. DUOXA1α and γ have three complete *N*-glycosylation sites, whereas DUOXA1-β and -∂ have only one glycosylation site that is insufficient to rescue oxidase activity.[43] Morand *et al.* examined the co-expression of DUOX1 or DUOX2 combined with DUOXA1-α, -γ or DUOXA2 in their transfection experiments into non-thyroid cells. The pairs of DUOX1–DUOXA1α and DUOX2–DUOXA2 generated H_2O_2 most effectively without producing O_2^- as expected. The heterogeneous pairs of DUOX1–DUOXA2 or DUOX2–DUOXA1-α and -γ are prominently expressed in the ER and produced O_2^-; they generated lower levels H_2O_2 at plasma membrane.[43]

Regulation of DUOX genes' expression and their enzymatic activity

TSH, cAMP, thyroid-specific transcription factors

Regulation of the DUOXs' expression is clearly different from that of the other thyroid-specific proteins of NIS, Tg and TPO. The DUOX2 mRNA is expressed without TSH in FRTL-5 rat cells or porcine thyrocytes, whereas NIS mRNA expression needs TSH stimulation.[44,45] The regulation of DUOX protein by TSH is much less than that of NIS and iodinated Tg.[46] In contrast, NOX4, another ROS-generating NADPH oxidase, is regulated by TSH.[22] Similarly, forskolin did not stimulate the transcriptional activities of human DUOX1 and 2 promoters or the DUOX protein expression in human thyrocytes, while it

activated the Tg and NIS promoters and TPO protein expression.[25] Pachucki *et al.*[25] reported that a thyroid-specific enhancer was not identified in the 5′-flanking regions of both DUOX genes. Thyroid transcription factor-1 and Pax8, well-known thyroid-specific transcription factors, do not appear to control DUOX1 and 2 promoter activities in PCCl3 rat cells.[47] The lack of thyroid-specific stimulants for DUOXs is in accord with their wide tissue distribution in the human body.

Cytokines

Cytokines can regulate the thyroid DUOXs' expression.[48,49] Th1-dominant cytokines (interleukin α [IL-1α] and interferon γ [IFN- γ]) and Th3-dominant cytokines (transforming growth factor β [TGF- β] and IL-10) decrease DUOX mRNA and its protein expression in human thyrocytes and rat thyroid cells.[48,49] Th2 cytokine, IL-4, reverses the inhibitory effects of IL-1 α, IFN- γ and IL-10, but not that of TGF-β.[48] Thus, autoimmune thyroid diseases, such as Th1-related Hashimoto's thyroiditis and Th2-dominant Graves' disease, may affect DUOX-mediated H_2O_2 production. The DUOX system in the respiratory tract epithelium has different cytokine regulation; Th1 cytokine, IFN-γ, highly induces DUOX2 mRNA, whereas Th2 cytokines, IL-13 and IL-4, increase DUOX1 mRNA.[50]

Ca^{2+}, protein kinase A and protein kinase C

As mentioned before, H_2O_2 generation is Ca^{2+}-dependent and it is activated not only by the increased level of intracellular Ca^{2+} but also by the phospholipase C-Ca^{2+} pathway.[8,51,52] Recently, Rigutto *et al.* described the differential regulation of DUOX1 and DUOX2 on H_2O_2 generation in transfected Cos-7 cells. The cAMP-dependent protein kinase A (PKA) stimulated DUOX 1, and protein kinase C (PKC) activated DUOX2 based on the phsophorylation of the respective DUOX proteins.[30] They also confirmed the above findings in human thyrocytes. Therefore, even though the main H_2O_2 producing machinery of DUOX2 fails, DUOX1-mediated H_2O_2 production may partially compensate as long as a cAMP signal prevails.[30]

Iodide

Iodide controls H_2O_2 generation in thyroid cells. Morand *et al.* have studied the effect of KI on H_2O_2 generation in porcine thyroid follicles, the most physiological thyroid culture system. They exposed follicles to 1 μmol/L KI for two days under cAMP stimulation and showed reduction in H_2O_2 production without affecting DUOX mRNA levels. Post-transcriptional change of the DUOX molecule by KI appears to be responsible for the decreased H_2O_2 generation.[53]

Hypothyroidism with DUOX2 and DUOXA2 gene mutation

Congenital hypothyroidism is the most common congenital endocrine disorder. The worldwide incidence is one in 4000 neonates. The most common cause of congenital hypothyroidism is thyroid dysgenesis (80–85%). Approximately 15–20% of infants with congenital hypothyroidism have goitrous hypothyroidism, suggesting dyshormonogenesis.[54] This disorder is caused by abnormalities of the thyrotropin (TSH) receptor, Gs protein, NIS, pendrin, TPO, Tg, PDS or DEHAL1.[55] Before the discovery of DUOXs and DUOXAs, there were two cases that might have been caused by abnormal H_2O_2 supply.[56,57] Now, cases of hypothyroidism due to DUOX2 or DUOXA2 mutation have been identified[15–21,41] (Tables 1 and 2).

Moreno *et al.*[15] reported the first four cases of DUOX2 mutation in 2002. Twenty-two patients with DUOX2 mutation have been reported.[15–21] Table 1 summarizes all reported cases of DUOX2 mutation and the clinical findings. There are 20 mutations in the DUOX2 gene: eight mutations in the extracellular peroxidase-like domain, 11 mutations in the first long intracellular loop and one mutation in the second intracellular loop (Figure 2). The types of mutation include missense mutation ($n = 8$), nonsense mutation ($n = 6$), frame shift ($n = 5$) and splice-site mutation ($n = 1$). Most cases were detected by the neonatal screening test for hypothyroidism, since both biallelic and monoallelic mutations resulted in hypothyroidism. Although all cases required thyroxine treatment initially, some of them were able to discontinue thyroxine treatment. According to Moreno *et al.*,[15] biallelic mutations lead to permanent hypothyroidism, whereas monoallelic mutations result in transient hypothyroidism. However, Maruo *et al.*[21] reported a transient hypothyroid case, despite biallelic mutations causing truncated DUOX2 protein. This report suggests the existence of an alternative mechanism to produce H_2O_2 when DUOX2 loses its function. The likely candidate is either DUOX1 or NOX4, since both molecules can be activated by TSH-PKA.[22,30] However, intact DUOX1 did not appear to function for the patient with biallelic mutation of DUOX2 reported by Moreno *et al.*,[15] and NOX4 is not localized on the apical membrane.[22] The alternative H_2O_2 generating system for DUOX2-DUOXA2 is still unclear.

The clinical phenotype of DUOX2 gene mutation may vary even though patients have the identical DUOX2 mutation as Vigone *et al.*[16] reported in two siblings. Their first sibling case had severe hypothyroidism and goiter, and his brother had mild hypothyroidism without goiter. The authors explained that the iodine supply could modify their phenotype. Also, our cases with the identical heterozygous mutation showed various phenotypes.[20] Thus, environmental factors including iodine intake may be responsible for variable phenotype expressions in this disorder.

The diagnosis of DUOX2 mutation is mainly based on the neonatal screening of thyroid function. Adult cases of DUOX2 mutation are difficult to diagnose unless physicians are aware of characteristics of dyshormonogenic goiter (marshmallow-like softness and enlarging goiter with age). Thyroid function tests vary from euthyroidism to hypothyroidism. To confirm iodide organification defect, a perchlorate discharge test has to be performed. This test is to demonstrate amount of radioactive iodine released from the thyroid gland after the administration of oral perchlorate. Radioactive iodine that does not bind to Tg (iodide organification defect) is released by perchlorate. The final

Table 1 List of reported cases with DUOX2 mutation

Authors	DUOX2 protein	No. of mutated allele	Age detected hypo/sex	Serum TSH before Rx (μIU/mL)	ClO_4^- discharge test (%) (<10)	Hypothyroidism	Information of carriers		
							Member	Gene mutation	Thyroid function
Moreno *et al.*[15] (2002), Netherlands	R434X	2	8D/F	1400	100	Permanent	Parents, brother	R434X hetero	Normal
	Q686X	1	10D/F	98	66	Transient	Father	Q686X hetero	Normal
	R701X	1	8D/F	26	41	Transient	Mother, brother	R701X hetero	Normal
	S965fsX994	1	7D/F	42	40	Transient	Father	S965fsX994 hetero	Normal
Vigone *et al.*[16] (2005), Italy	R376W/R842X	2	7D/M	173.2	28	Permanent	Father	R376W hetero	Normal
			11D/M	9.6	12	Permanent	Mother	R842X hetero	Normal
Varela *et al.*[17] (2006), Argentine	Q36H/S965fsX99	2	3D/M	156	46	Permanent	Mother	Q36H hetero	Normal
							Father	S965fsX994 hetero	Normal
	G418fsX482	2	8M/F*	>100	68	Permanent	Mother*	G418fsX482 hetero	Sub hypo
	IVS19-2A > C		1M/M	>100	60	Permanent	Father*	IVS19-2A > C, hetero	Normal
							Brother,* sister, son	IVS19-2A > C, hetero	Sub hypo
Pfarr *et al.*[18] (2006), Germany	Ins602g > fsX300	1	3D/F	250	ND	To be determined	Mother	Ind602g > fsX300, hetero	Normal
	Ins602g > fsX300/D506N	2	3D/F	>150	ND	To be determined	Father	Ind602g > fsX300, hetero	Normal
							Mother, brother	D506N, hetero	Normal
DiCandia *et al.*[19] (2006), Italy	S965fsX994	1			20–63	To be determined			
	Q1026X	1			20–63	To be determined			
Ohye *et al.*[20] (2008), Japan	R1110Q	2	54Y/F	4.36	72.8	Permanent	Son	R1110Q, hetero	Normal
							Son, grandson, granddaughter†	R1110Q, hetero	Sub hypo
Maruo *et al.*[21] (2009), Japan	L479SfsX481/K628fsX638	2	9D/F	95.4	ND	Transient	Mother	K628RfsX638, hetero	
			6D/F	233.0					
			6D/F	150			Father	L479SfsX481, hetero	
			6D/M	25.7					
	K530X/E879K&L1067S‡	2	14D/F	41.6	ND	Transient	Mother	E879K&L1067S‡, hetero	
			9D/F	18.9			Father	K530X, hetero	
	H678R/L1067S‡	2?	5D/F	98.5	ND	Transient	Mother	L1067S‡, hetero	
							Father	H678R, hetero	
	A649E/R885Q	2	17D/F	24.8	ND	Transient	Mother	A649E, hetero	
							Father	R885Q, hetero	

TSH, thyrotropin; TPO, thyroid peroxidase; TgAb, thyroglobulin antibody
*Positive anti-TPO or TgAb; †T4 treatment since neonate; ‡Potential benign polymorphism

Table 2 List of reported case with DUOXA2 mutation

Authors	DUOXA2 protein	No. of mutated allele	Age detected hypo/sex	Serum TSH before Rx (μIU/mL)	ClO_4^- discharge test (%) (<10)	Hypothyroidism	Information of carriers		
							Member	Gene mutation	Thyroid function
Zamproni and Grasberger *et al.* (2008)[41]	Y246X	2	<43D/F	12–102	18	Permanent	Mother, father, sisters	Y246X, hetero	Normal

TSH, thyrotropin; TPO, thyroid peroxidase

diagnosis can be made by analyzing gene mutation. Thyroxine treatment should be started in the neonatal period and continued for the first several years to enhance normal development. It is necessary to confirm whether the hypothyroidism is permanent or transient after several years of thyroxine treatment. Even though thyroxine treatment is withheld for transient hypothyroidism, thyroid function has to be periodically examined.

Grasberger *et al.*[40] carried out the functional study of DUOX2 mutants in Cos-7 cells. They analyzed the following three missense mutations of the peroxidase-like domain (Q36H, R376W and D506N). The Q36H and R376W mutations completely abolished the transition of DUOX from the ER to the plasma membrane. Also, the D506N mutant reduced its plasma membrane translocation. These DUOX2 mutants were retained within the ER. They concluded that post-transitional processing of the peroxidase-like domain plays a crucial role for the ER exit of DUOX2. The mutation site at the R1110Q reported by Ohye *et al.*[20] corresponds to the highly conserved arginine residues of R80 in the NOX2, an NADPH oxidase of phagocytes. Interestingly, R80E mutation resulted in complete abolishment of ROS production in the NOX2 molecule because of failure to form a heterodimer with integral membrane protein $p22^{phox}$, a docking site for the regulatory proteins.[31] It is speculated that the R1110 has a function similar to the R80 of NOX2.

Zamproni *et al.*[41] first described a patient with mild congenital hypothyroidism due to the homozygous nonsense mutation (Y246X) in the DUOXA2. The patient had a goiter with a partial iodide organification defect. The clinical features are summarized in Table 2. The mutation is located at the second extracellular loop (Figure 3) and produces a truncated DUOXA2 protein lacking the fifth transmembrane domain and C-terminal. The transfection of the Y246X mutant into Hela cells failed the translocation of DUOX2 to the cell surface with no detectable H_2O_2. Also, DUOXA1 could not substitute for abnormal DUOXA2 in their cross-functioning study.[41]

ROS-mediated thyroid diseases

Thyroid cancer

Lacriox *et al.* examined DUOX1 and DUOX2 mRNA levels from thyroid cancer tissues and normal thyroid tissues obtained from patients, whose thyroid function tests were normal at the time of surgery. Thyroid cancer tissues

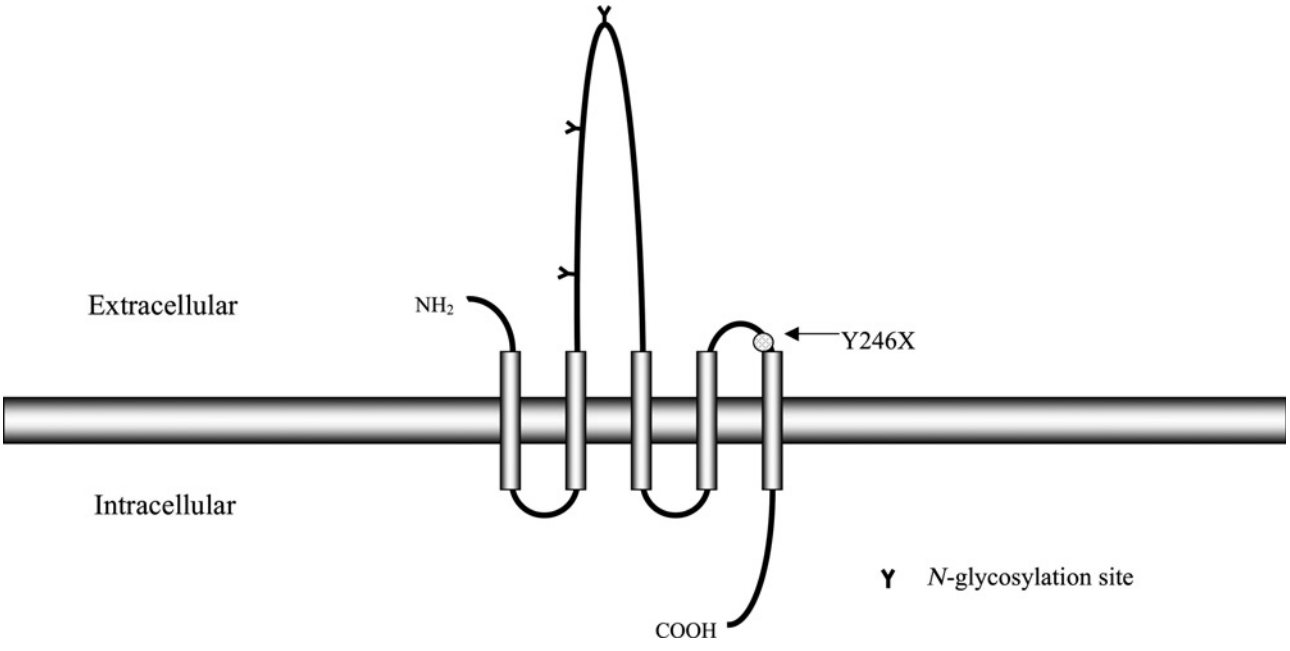

Figure 3 Structural model of DUOXA2 protein and mutation site. A small closed circle indicates the location of mutation and changes in amino acid

displayed a wide variation of DUOX1 and DUOX2 mRNA levels. The mean DUOX1 and DUOX2 mRNA levels of cancer tissues did not differ from those of normal thyroid tissues. They also examined the DUOX1 and DUOX2 protein expression by immunostaining. The variable levels of DUOX protein expression were seen in thyroid cancer tissues.[58] Also, abolished or decreased H_2O_2 generation was detected in some of the thyroid carcinoma tissues by others.[59] The discrepancy between DUOX expression levels and H_2O_2 generation seen in some cancer tissues is probably caused by different localizations of the DUOX protein either in the apical membrane or in the cytoplasm.[58,60,61] One interesting aspect is that the majority of cancer tissues exhibited normal or slightly increased DUOX protein expressions while NIS, TPO and PDS protein expressions were profoundly decreased or absent.[58]

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, and radiation is the only proven cause. There is a growing speculation that H_2O_2 can be the cause of sporadic PTC.[62] Detours *et al.*[62] found that Chernobyl Tissue Bank (PTC after radiation exposure) and French sporadic PTC (no radiation exposure) had similar overall gene expression profiles, RET/PTC rearrangements and BRAF mutation rates. Also, transcriptional responses of human lymphocytes to 200 μmol/L H_2O_2 and 2.5 Gy radiation had a significant similarity. Therefore, sporadic PTC and radiation-induced PTC appear to be the same disease category, although they are distinguishable with molecular signatures. Maintenance of normal or slightly increased thyroid DUOX protein expression in thyroid cancer,[58] long-term H_2O_2 exposure due to 8.5 years' life span of thyroid cells,[63] membrane permeable nature of H_2O_2 and decreased TPO expression in cancer tissues (less utilization of H_2O_2) should partly explain the accumulated DNA damage by H_2O_2 and its possible contribution to the development of PTC. In addition, Weyemi *et al.*[22] recently provided convincing evidence of activation of intracellular ROS by NOX4 in human PTC tissues. Furthermore, the same authors described activation of $p22^{phox}$, an active partner of NOX4, by H-Ras oncogene in cultured human thyrocytes.[64]

Hashimoto's thyroiditis

Hashimoto's thyroiditis is a common thyroid disease for women and involves genetic, immunological and environmental factors for its development. The most important environmental factor is iodine. A survey in China showed an increased prevalence of Hashimoto's thyroiditis and hypothyroidism with excessive iodine intake.[65] A key question is: how does increased iodide ingestion contribute to the development of Hashimoto's thyroiditis. One of the important aspects of iodide, besides thyroid hormone formation, is the Wolff–Chaikoff effect in the thyroid gland. This protects thyrocytes from acute iodide overload by inhibiting iodide organification and by discharging iodide recently transported into the thyroid gland. Therefore, administration of a large amount of iodide is a standard procedure to prevent ^{131}I-iodine accumulation in the thyroid gland from nuclear accidents. The Wolff–Chaikoff effect is caused by iodide-mediated inhibition of H_2O_2 generation.[51] The study of NOD.H2^{h4} mouse, an animal model of Hashimoto's thyroiditis, provides us with insight into the understanding of iodide handing in susceptible host.[66,67] Exposure of NOD.H2^{h4} thyroid cells to 100 μmol/L iodide (more than 100-fold higher iodide concentration than physiological dose) increased extracellular H_2O_2 production that subsequently activated intracellular adhesion molecule 1 (ICAM-1) expression. Constitutive expression of ICAM-1 in the thyroid cell of NOD.H2^{h4} mouse and ICAM-1 activation by H_2O_2 results in the retention of circulating lymphocytes and subsequent cytokine release favoring the development of Hashimoto's thyroiditis.[66] The high level of H_2O_2 also produces the antigenic antigen from oxidation damaged iodinated Tg.[68] Antioxidants blocked the generation of ROS and ICAM-1 expression. In a non-susceptible mouse, 100 μmol/L iodide did not generate H_2O_2.[66,67] The animal experiments suggest that susceptible hosts have defective Wolff–Chaikoff effect allowing them to generate H_2O_2 in response to increased iodide influx, which ordinarily should not happen as seen in the non-susceptible mouse.[67] Thus, abnormality of thyroid H_2O_2 generation in response to high iodide may play a role in the development of Hashimoto's thyroiditis in susceptible individuals. Whether iodide-mediated H_2O_2 generation is driven by activated DUOX or NOX4 or defective antioxidants has yet to be studied.

Multinodular goiter or hypofunctioning adenoma

The levels of mRNA expression of the DUOXs are highly variable; no statistical difference was noted between nodular tissues and normal tissues. The DUOXs' protein expressions in multinodular goiter and hypofunctioning adenoma are also highly variable from one follicle to the other.[35,60] However, Caillou *et al.*[35] found a higher number of positive DUOX cells in hypofunctioning follicular adenoma than in hyperfunctioning tissue. This finding suggests that DUOX protein expression does not always correlate with the functional state of thyroid tissue. The other common feature was weak staining in macrofollicular area and flattened cells, whereas strong staining is seen in the microfollicular area and cuboidal cells, an index of active thyroid cells.[35] This trend is also found in normal thyroid tissue.[69] NOX4 immunostaining was also found in the cytoplasm in multinodular thyroid tissue.[22] However, the possible contribution of DUOX or NOX4 proteins to the development of multinodular goiter or adenoma deserves further investigation.

Hyperthyroidism

In hyperfunctioning thyroid tissue, there are increased iodinated Tg, NIS and TPO protein expressions with slightly increased PDS expression contributing to excess thyroid hormone production.[60,61] However, the DUOXs' expression is variable. The DUOXs' mRNA expression was relatively low in thyroid tissues of Graves' disease and varied widely in toxic adenoma when compared with that of normal tissue. In the immunostaining, only 0–10% of

follicular cells was positive for DUOX protein in the hyperfunctioning thyroid.[35] Other investigators described slightly increased DUOXs' protein levels in hyperfunctioning thyroid tissues.[60] One potential explanation for these variable expression levels is the effect of the treatment with anti-thyroid drugs before surgery. Methimazole and propylthiouracil inhibit NADPH oxidase activity.[70] Some of the hyperfunctioning nodules did not express DUOXs' protein after anti-thyroid drug treatment.[60] Interestingly, an impairment of iodide organification in hyperactive portions of the thyroid has been reported.[71,72] Roti *et al.*[71] reported that 69% of the 29 patients with untreated Graves' disease showed a positive perchlorate discharge test, and this abnormality was corrected with the anti-thyroid drug treatment for more than 50% patients. Thyroid-stimulating antibody found in patients with Graves' disease does not appear to stimulate H_2O_2 generation.[73] Moreno-Reyes *et al.*[72] also described a positive perchlorate discharge test in 61% of 46 patients with autonomous thyroid nodules. H_2O_2 generation in autonomous thyroid nodules is lower than in the corresponding normal thyroid tissue.[74] Taken together, an impaired iodide organification due to low H_2O_2 generation can possibly happen in some patients with untreated hyperfunctioning thyroid. The involvement of other members of NOX family has to be investigated to establish the H_2O_2 generation status in hyperthyroid thyroid glands.

Conclusions

The DUOX2–DUOXA2 system has been known as the extracellular source of H_2O_2 in the thyroid gland. Now, NOX4 is a newly added source of intracellular ROS in the thyroid gland.[22] Mutation of either DUOX2 or DUOXA2 causes hypothyroidism due to insufficient H_2O_2 generation. We have summarized clinical and molecular aspects of all DUOX2 and DUOXA2 mutations from patients so far reported (Tables 1 and 2, Figures 2 and 3). Thyroid cells may accumulate oxidative damage due to constant exposure to H_2O_2 and other ROS. The imbalance between ROS production and its degradation systems[74] may create an oxidant dominant state causing sporadic PTC[22,62] or Hashimoto's thyroiditis.[66,67] Our present review suggests that the thyroid H_2O_2 generating systems might be involved, not only in the hypothyroidism caused by DUOX defects but also in some other common thyroid diseases.

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