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Role of the NADPH Oxidases DUOX and NOX4 in Thyroid Oxidative Stress

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Abstract
Somatic mutations are present at high levels in the rat thyroid gland, indicating that the thyrocyte is under oxidative stress, a state in which cellular oxidant levels are high. The most important class of free radicals, or reactive metabolites, is reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2). The main source of ROS in every cell type seems to be mitochondrial respiration; however, recent data support the idea that NADPH:$O_{(2)}$ oxidoreductase flavoproteins or simply NADPH oxidases (NOX) are enzymes specialized in controlled ROS generation at the subcellular level. Several decades ago, high concentrations of H_2O_2 were detected at the apical surface of thyrocytes, where thyroid hormone biosynthesis takes place. Only in the last decade has the enzymatic source of H_2O_2 involved in thyroid hormone biosynthesis been well characterized. The cloning of two thyroid genes encoding NADPH oxidases dual oxidases 1 and 2 (DUOX1 and DUOX2) revealed that DUOX2 mutations lead to hereditary hypothyroidism in humans. Recent reports have also described the presence of NOX4 in the thyroid gland and have suggested a pathophysiological role of this member of the NOX family. In the present review, we describe the participation of NADPH oxidases not only in thyroid physiology but also in gland pathophysiology, particularly the involvement of these enzymes in the regulation of thyroid oxidative stress.

Introduction

The thyroid follicle is the functional structure responsible for thyroid hormone biosynthesis, storage and secretion (fig. 1). Follicular thyroid cells are polarized and specialized in the production of thyroxine (T4) and 3,3′,5-triiodothyronine (T3). Cellular function thus depends on the expression of a set of well-characterized proteins involved in hormone biosynthesis [1].

Thyroid hormones are synthesized within the structure of a high-molecular-weight, thyroid-specific protein called thyroglobulin, and they remain covalently bound to the primary structure of this molecule until protein degradation occurs and the hormone is secreted. The first step in thyroid hormone biosynthesis corresponds to the iodination of tyrosyl residues of thyroglobulin.

Thyroglobulin is synthesized and secreted into the follicular lumen, and the iodination reaction is believed to occur at the apical membrane of the cells and the colloid interface [2]. Hence, at the apical surface of the thyrocyte

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(fig. 1), a number of chemical reactions are fundamental for thyroid hormone synthesis: (1) iodide oxidation; (2) tyrosyl radical oxidation; (3) thyroglobulin iodination, namely iodine organification, and (4) coupling of iodotyrosines to form the iodothyronines, mainly T4 and T3 [1].

The enzymatic reactions that involve the oxidation of substrates depend on the presence of hydrogen peroxide (the oxidant substance) and a peroxidase enzyme that catalyses the process, thyroperoxidase (TPO). In the thyrocyte, the hydrogen peroxide necessary for hormone biosynthesis is generated at the apical surface of the cell through a controlled reaction catalyzed by NADPH:$O_{(2)}$ oxidoreductase flavoproteins [3] or simply NADPH oxidases, the dual oxidases DUOX1 and DUOX2. These enzymes are members of the NADPH oxidase (NOX) family of oxidoreductase enzymes, and DUOX1 shares 83% similarity with the DUOX2 gene.

Some decades ago, Bjorkman and Ekholm [4] detected H_2O_2 at the apical surface of thyrocytes, indicating a relatively high level of this oxidizing agent in the thyroid gland. More recently, the observation that somatic mutations are present in higher levels in the rat thyroid gland has further confirmed that the thyrocyte is under oxidative stress [5]. However, the source of thyroid H_2O_2 involved in hormone biosynthesis and cell damage has only recently begun to be clarified. In the present review, we describe the role of NADPH oxidases not only in thyroid physiology but also in gland pathophysiology.

Thyroid Sources of Hydrogen Peroxide and the NADPH Oxidases

In the 1970s, various studies were performed with the aim of characterizing the source of hydrogen peroxide associated with thyroperoxidase. Among the enzymatic systems proposed were NADPH-cytochrome c reductase [6], NADH-cytochrome b5 reductase [7], monoamine oxidase [8], and xanthine-xanthine oxidase [9]; however, the inhibition of these enzyme activities did not correlate with organification blockage. Moreover, these enzymes are located in subcellular compartments other than the apical plasma membrane, thus calling into question their involvement in hormone biosynthesis; this reaction has been demonstrated to occur at the apical surface of thyrocytes, at the cell-colloid interface, as suggested by histological findings [4]. In 1984, Virion et al. [3] described an enzymatic system that generated H_2O_2 in a calcium- and NADPH-dependent manner and was located at the thyrocyte cell membrane. This type of enzymatic system was strongly suggested in the early seventies, even before the histological description of the presence of H_2O_2 at the apical surface of thyrocytes [10]. The report by Virion et al. [3] was followed by several other demonstrations of the biochemical properties of the enzyme [11–15], but no physiological role was proven. Later, two independent groups published reports indicating that thyroid hydrogen peroxide generation [16] and the calcium-dependent NADPH oxidase activity [17] in primary cell culture models were both dependent on the presence of thyrotropin in the medium. These reports further reinforced the idea of a pivotal role of the calcium-dependent NADPH oxidase in thyroid hormone biosynthesis.

In 1999, Dupuy et al. [18] cloned a thyroid oxidase gene that was predicted from the purified NADPH oxidase protein sequence. In parallel, the genes of two NADPH oxidases were cloned from thyroid cDNA libraries [19]. These genes were first named thyroid oxidases (THOX) because they were believed to be thyroid specific [20]. However, both the THOX1 and THOX2 genes are expressed in tissues other than the thyroid. The sequences of both proteins predict an extracellular peroxidase-like domain, apart from the intracellular calcium-binding EF-hand motifs and NADPH binding site. Thus, the nomenclature was changed to DUOX1 and DUOX2 to reflect that they are dual oxidases due to their different structures when compared to the other NADPH oxidases (NOX) that do not have the extracellular peroxidase-like domain in their structures. Presently, the NOX family is composed of seven members: five NOXes (NOX1, NOX2, NOX3, NOX4, and NOX5) and two DUOXes (DUOX1 and DUOX2). All NOX family members have six transmembrane domains containing the two heme-binding regions, and a long cytoplasmic C-terminus that contains the FAD and the NADPH-binding regions [21].

Dual Oxidases: DUOX1 and DUOX2

Under normal conditions, dual oxidases are highly expressed in the thyroid gland, salivary gland, gastrointestinal tract, respiratory epithelia and breast tissue [21, 22]. However, the thyroid gland is the only tissue where both DUOX1 and DUOX2 are expressed under physiological conditions. The definitive proof that DUOX is fundamental for thyroid hormone biosynthesis can be found in the first description of mutations in DUOX2 genes related to human goiter and hypothyroidism [23]. Before these findings were reported, however, seminal studies that characterized the calcium-dependent NADPH oxi-

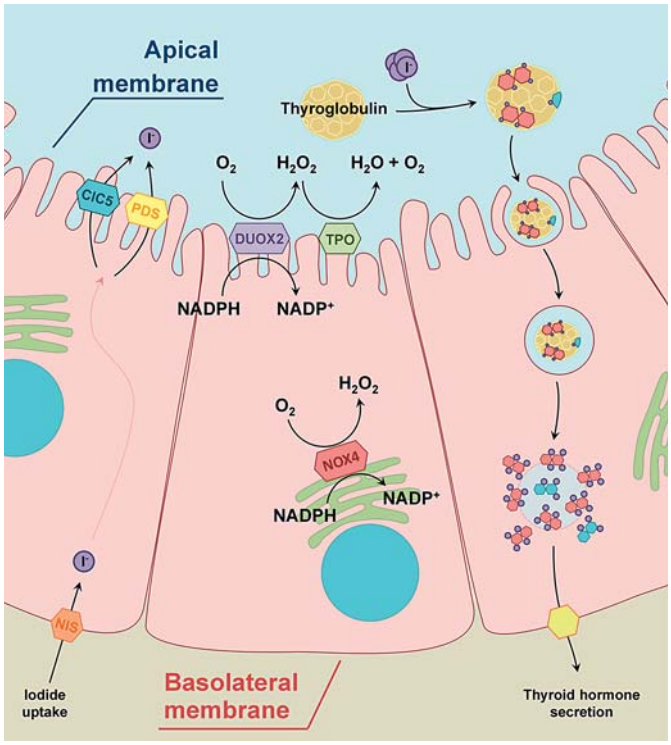
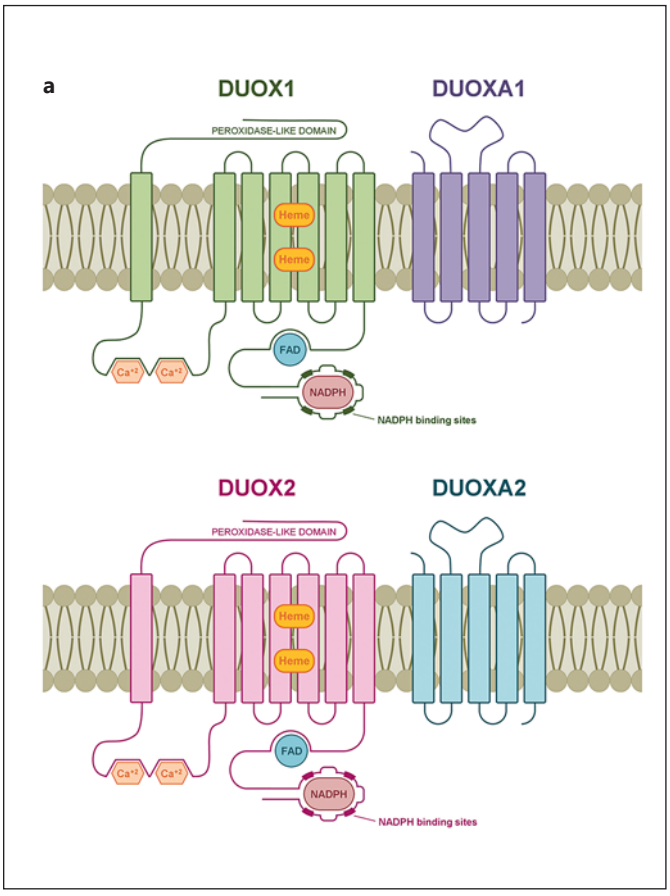


Fig. 1. Schematic representation of the thyroid follicle. The basolateral cell membrane is in close contact with the circulation, and the apical cell membrane faces the follicular lumen, which is the site of thyroid hormone biosynthesis.



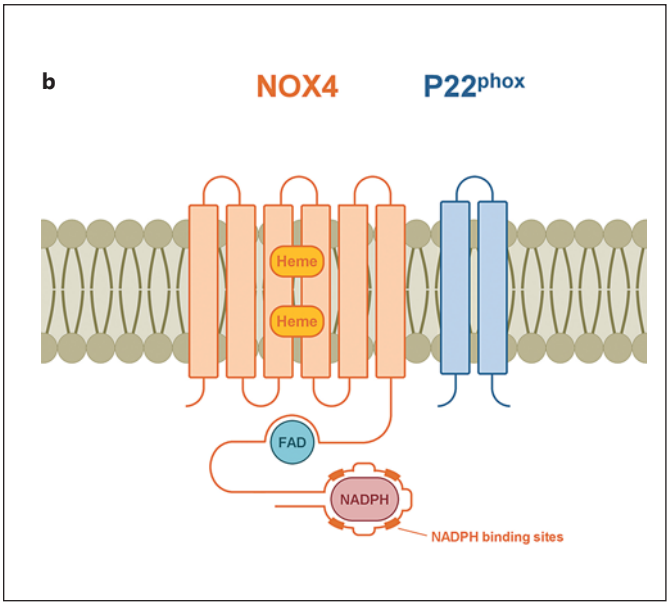


Fig. 2. **a** Schematic representation of DUOX1 and DUOX2 and their activators DUOXA1 and DUOXA2. **b** Representation of NOX4 and its partner p22Phox.

dase activity in the human thyroid [24] were fundamental to the description of biochemical defects in the thyroid calcium-dependent NADPH oxidase activity in goiter and hypothyroidism [25], as well as the blockage of iodine organification that occurs after iodine overload, known as the Wolff-Chaikoff effect [26–28]. These reports had already suggested that the enzymatic system related to thyroperoxidase and hormonogenesis was in fact dependent on calcium and NADPH.

Although other studies had confirmed by 2002 that human mutations in the DUOX2 gene are related to thyroid dyshormonogenesis, no functional analyses could be performed because even the wild-type enzyme did not function in heterologous transfected cells. It was then proposed that DUOX retention in the endoplasmic reticulum of heterologous systems could be due to the lack of an unidentified component required for the functionality of the enzyme. After 2006 [29], with the cloning of the gene of its essential partner, DUOXA, in vitro functional studies could be performed and our understanding of the thyroid H_2O_2 generating system began to be elucidated (fig. 2a).

The first interesting finding was related to the fact that both the DUOX1 and DUOX2 genes are organized head-to-head on human chromosome 15 and are coexpressed with the genes of their partners or maturation factors: DUOXA1 and DUOXA2, respectively [29]. Grasberger and Refetoff [29] demonstrated that coexpression of DUOXA2 allowed ER-to-Golgi transition, maturation, and translocation of functional DUOX to the plasma membrane. This study not only improved the understanding of the molecular genetics of hypothyroidism related to DUOX and DUOXA mutations but also led to functional studies that better elucidated the participation of DUOX in thyroid hormonogenesis and its interaction with thyroperoxidase [30–35].

The first report on the functional impact of the DUOX2 mutations found in patients with congenital hypothyroidism described complex enzymatic system regulation, showing that DUOX missense mutations might impair its interaction with DUOXA and its trafficking to the cell membrane, which might culminate with DUOX2 degradation [30]. In fact, the majority of DUOX2 or DUOXA2 biallelic mutations have been described in patients with permanent congenital hypothyroidism, while other studies report cases of transient congenital hypothyroidism due to heterozygous nonsense or frameshift mutations in DUOX2 or DUOXA2 [31, 32]. Interestingly, there is also a report of transient hypothyroidism in patients with early frameshift mutations in the DUOX2 gene (mutations that most likely lead to completely inactive proteins) [31]. In animal models, the absence of DUOXA genes leads to a severe hypothyroid phenotype [33], similar to that which occurs in mice with a DUOX2 missense mutation [36].

To date, no human DUOX1 mutations related to thyroid dyshormonogenesis have been identified, and the exact role of this enzyme in thyroid physiology remains elusive. However, because some DUOX2 and DUOXA2 mutations lead to transient hypothyroidism, it is believed that DUOX1 could function to overcome the lack of DUOX2 activity, at least under some still undefined circumstances.

Recently, using site directed mutagenesis of DUOX2, the importance of cysteine residues in the peroxidase-like extracellular domain has been determined [37]. Interestingly, it was demonstrated that thyroperoxidase activity is inhibited by H_2O_2 generated by DUOX, most likely due to oxidative damage to the enzyme, which could lead to higher cell oxidative stress [37]. These findings are in accordance with a previous report of a negative correlation between TPO and H_2O_2 generating activities in human goiters [38]. Increased DUOX activity, along with reduced TPO activity, could have deleterious effects on the thyroid tissue because H_2O_2 is not only produced in higher amounts by DUOX but is also less consumed by TPO. Reactive oxygen species could then accumulate, leading to oxidative damage of the thyroid gland [39–41].

It is now tempting to speculate as to whether other molecules might interact with the DUOX-DUOXA complex and regulate thyroid hormonogenesis and oxidative stress. A previous report showing that NOXA1 interacts with DUOX in a mucociliary airway epithelium model and downregulates its H_2O_2 generating activity provided new insight into the regulation of this complex enzymatic system [42].

The regulation of H_2O_2 generation and DUOX/DUOXA gene expression is quite complex. Although a tonic modulation by thyrotrophic hormone through the cAMP pathway seems to be fundamental for the enzyme to be expressed in primary cell culture models [16, 17], the tonic in vivo increase in cAMP might somehow regulate the DUOX enzymes in a different manner. In the mouse thyroid, DUOX proteins were found at embryonic day 15.5 and were mainly localized at the apical pole of thyrocytes, confirming their important role in hormonogenesis. Interestingly, tonic in vivo TSH stimulation of the gland led to a decrease of H_2O_2 generation without affecting DUOX expression in mice [43]. Additionally, a substantial decrease in DUOX immunostaining in human hyperfunctioning tissues (Graves' diseases and toxic adenoma) has been observed, demonstrating the existence of mechanisms that may protect hyperstimulated thyrocytes from oxidative stress [44]. More recently, using a heterologous model, it has also been shown that the basal activity of both DUOX isoenzymes in fact depends on calcium [45–47]. However, DUOX1, but not DUOX2, activity is acutely stimulated by forskolin via protein kinase A-mediated DUOX1 phosphorylation on serine 955, while phorbol esters induce DUOX2 phosphorylation via protein kinase C activation associated with high H_2O_2 generation [47]. Although the cAMP pathway can acutely phosphorylate DUOX1, increasing its activity, our group has recently shown that the long-term effect of PKA activation seems to be the reduction of DUOX1 expression and activity in PCCL3 cells [48]. Therefore, data on DUOX regulation are somewhat contradictory and appear to depend on the animal model studied, the concentration of TSH and the period of treatment. More recently, it has been demonstrated that physiological concentrations of thyroglobulin inhibit the expression and function of DUOX2 in thyroid cells [49]. Thus, thyro-

globulin content in thyroid follicles might be involved in the regulation of H_2O_2 production by individual follicles, as has been reported for iodine [28].

Importantly, in diabetic rats, thyroid DUOX1 gene expression is upregulated, and our laboratory has demonstrated that in PCCL3 thyroid cells, the DUOX1 increase is directly mediated by glucose through the protein kinase C pathway [48], indicating a pathophysiological role for this enzyme in the thyrocyte under stress. In addition to DUOX1 regulation by glucose, we have also reported that NOX4 gene expression is increased in the thyroid of diabetic rats [48].

NOX4

NOX4, another NOX family member (fig. 2b), was recently described in human thyrocytes [50], but its role in thyroid pathophysiology remains to be further elucidated.

In contrast with DUOX (fig. 2a), NOX4 generates H_2O_2 and/or O_2^- in intracellular compartments such as the endoplasmic reticulum, mitochondria or nucleus [51–53], and it is constitutively active [54]. DUOX1 and DUOX2 are active in the cellular plasma membrane only in the presence of DUOX maturation factors, and DUOX activation depends on the rise of intracellular calcium levels. On the other hand, NOX4 seems to be fully active in the presence of p22phox or possibly Poldip2 proteins, but enzyme activity is constitutive, depending only on its expression levels.

It was demonstrated that NOX4 and p22phox expression are upregulated in thyroid cancers, linking NOX4-dependent H_2O_2 generation to cancer development or progression [49, 54–56]. In contrast, DUOX2 expression is normal or even decreased in the majority of the differentiated thyroid carcinomas [57, 58].

More recently, our group has shown that NOX4 mRNA expression is higher in adult female rat thyroids [59]. This sexual dimorphism was not found in prepubertal thyroids, indicating a direct role of sexual steroids in thyroid NOX4 expression control. Consistent with this hypothesis, we have shown that in proestrus, the estrous cycle phase that is characterized by a peak in serum estrogen, NOX4 mRNA levels were higher in comparison to the other phases, and higher thyroid H_2O_2 production was detected in the estrus phase. Moreover, NOX4 and Poldip2 mRNA levels, as well as the H_2O_2 production rate, were higher in PCCL3 cells incubated with 17β-estradiol.

Weyemi et al. [50] reported that in normal human thyroid tissue, NOX4 immunostaining is intracytoplasmic. Although Fortunato et al. [59] confirmed that NOX4 immunostaining in the thyroids of both male and female rats is intracytoplasmic, we have also detected NOX4 immunostaining at the plasma membrane of thyrocytes. This difference suggests a species-specific regulation of the subcellular distribution of thyroid NOX4.

H_2O_2 has been linked to various processes related to the development of thyroid nodules and cancer, such as cellular proliferation, DNA damage and apoptosis inhibition. Salmeen et al. [60] recently showed that NOX4 and DUOX2 are involved in the cell cycle entry of a fibroblast cell lineage through inactivation of the p53-dependent checkpoint pathway. These authors suggest that the inactivation of p53 could be due to oxidation of its cysteine residues by DUOX2 and NOX4 generated H_2O_2, decreasing its DNA binding activity. Moreover, some protein tyrosine phosphatases have catalytic cysteine residues that can be oxidized by reactive oxygen species (ROS), leading to their inactivation [61].

All of the H_2O_2 actions described above reinforce the hypothesis that female thyroids are chronically exposed to higher levels of H_2O_2 compared to males and thus have greater potential to cause macromolecular damage, a higher cell proliferation rate and inhibition of apoptosis, all of which might be associated with increased risk of thyroid disorders. In fact, there is recent evidence confirming a gender disparity in thyroid cancer, corroborating our hypothesis [62].

Deregulation of NOX4 expression and/or activity in the nuclear region may create a threat to DNA stability and influence the cell fate. Using doxycycline-inducible expression of H-RasV12, we provided the first evidence that oncogene-induced DNA damage was mediated by increased ROS generation via NOX4 upregulation [56]. As ROS are known to promote tumorigenesis and other age-related diseases [63], NOX4 would seem to be a potential target for mitigating levels of DNA lesions driven by various stimuli.

Our novel findings of NOX4 expression in the thyrocyte might contribute to elucidating its role in the molecular basis of thyroid disease.

Conclusions

On the apical membrane of thyrocytes, H_2O_2 acts as a TPO cosubstrate in thyroid hormone biosynthesis. The sources of thyrocyte H_2O_2 related to hormonogenesis are

the DUOX enzymes, DUOX1 and DUOX2, which have two calcium-binding sites that are indispensable for their activities. Another NOX family member, NOX4, has recently been described in thyrocytes and, in contrast with DUOX, it seems to mainly generate ROS in the intracellular compartment and is constitutively active.

Apart from its role in thyroid hormonogenesis, H_2O_2 is normally produced by all cells of living organisms, and it is able to act in the redox-dependent regulation of different cellular functions, including response to stressors, angiogenesis, and cell proliferation, among others. Conceptually, an imbalance between pro-oxidant compounds and antioxidant defenses leads to the disruption of redox signaling and control. Thus, H_2O_2 has been linked to various processes related to the development of thyroid nodules and cancer, such as cellular proliferation, DNA damage and apoptosis inhibition. As a result, the enzymes involved in thyroid ROS generation might be implicated not only in thyroid physiology but also in gland pathophysiology in a gender-specific manner. Thus, the mechanisms underlying the regulation of thyroid ROS generation and detoxification need to be further clarified.

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Disclosure Statement

The authors have nothing to disclose.

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